STUDIES ON THE MICROSPORIDIA OF INSECT PESTS OF MULBERRY AND OTHER AGRICULTURAL CROPS

THESIS

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Submitted By

MUKTA MAYEE KUMBHAR

Under the supervision of

Dr. SUMAN MISHRA
Assistant Professor

BABASAHEB
BHIMRAO
AMBEDKAR
UNIVERSITY
LUCKNOW
प्रज्ञा शील करुणा
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BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
(A CENTRAL UNIVERSITY)
VIDYA VIHAR, RAEBARELI ROAD, LUCKNOW-226 025 (U.P.), INDIA

Enrolment No.118/12 **Year 2017**

Dedicated to

My Beloved Family Members

DECLARATION

It is hereby declared that, the present thesis entitled **"Studies on the microsporidia of insect pests of mulberry and other agricultural crops"** submitted to the Department of Applied Animal Sciences, School for Biosciences and Biotechnology, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow for the award of Ph.D. degree is a record of original work done by me under the supervision of Dr. Suman Mishra. The matter embodied in this Ph.D. thesis has not been submitted in this university or any other university for the award of any other degree or diploma.

Mukta Mayee Kumbhar

Place: Lucknow
Date: 18/08/2017

Mukta Mayee Kumbhar
Enrolment No: 118/12

CERTIFICATE

This is to certify that, the thesis titled **"Studies on the microsporidia of insect pests of mulberry and other agricultural crops"** submitted by **Ms./Mr. Mutka Mayee Kumbhar** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University Lucknow satisfies all the requirements as stipulated in the *Doctor of Philosophy (Ph.D.) regulations - 1999 as amended in 2008/2010/2013* and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

Date: 18.08.207

18/08/2017
Supervisor

18/8/17
Head of the Department

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Date - 18/08/2017
Place- Lucknow

Mukta Mayee Kumbhar

Mukta Mayee Kumbhar
Enrollment No- 118/12
Department of Applied Animal Sciences
Babasaheb Bhimrao Ambedkar University,
Lucknow-226025, U.P.

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ABBREVIATION

S. NO.	ABBREVIATION	FULL NAME
1.	min	Minute
2.	nm	Nanometer
3.	µm	Micrometer
4.	cm	Centimeter
5.	m	Meter
6.	g	Gram
7.	kgs	Kilograms
8.	%	Percentage
9.	K_2CO_3	Potassium carbonate
10.	$C_5H_8O_2$	Glutaraldehyde
11.	OsO_4	Osmium tetroxide
12.	ml	Milliliter
13.	M	Molar (moles/liter)
14.	AD	Anchoring disc/ Adhesive disc
15.	EN	Endospore
16.	EX	Exospore
17.	PF	Polar filament
18.	PP	Polaroplast
19.	N	Nucleus
20.	PV	Posterior Vacuole
21.	LC50	Median lethal concentration
22.	SEM	Scanning Electron Microscopy
23.	TEM	Transmission Electron Microscopy
24.	v/v	Volume/volume
25.	PI	Post inoculation
26.	M-Dch	Microsporidia isolated from *Danaus chrysippus*
27.	M-Cfl	Microsporidia isolated from *Catopsilia florella*
28.	M-Mph	Microsporidia isolated from *Melanitis phedima*
29.	M-Ame	Microsporidia isolated from *Apis mellifera*

30.	M-Scy	Microsporidia isolated from *Samia cynthia ricini*
31.	M-Nbo	Microsporidia isolated from *Nosema bombycis*
32.	sp.	Species
33.	SR	Shell ratio
34.	SD	Standard deviation
35.	rpm	Rotation per minute
36.	pH	Measure of acidity or alkalinity of a solution
37.	^{o}C	Degree centigrade
38.	KV	Kilovolt
39.	B.C.	Before Christ
40.	AD	Anno domini,
41.	MT	Metric Ton
42.	dfls	Disease Free Layings
43.	ha	Hectare
44.	R.H.	Relative humidity
45.	kg/ha/yr	kilogram / hectare / year
46.	Lbms	Microsporidia is associated with the Lamerin breed of the silkworm, *Bombyx mori* L.
47.	Bm CPV	*Bombyx mori* Cytoplasmic Polyhedrosis Virus
48.	Bm DNV	*B. mori* Densonucleosis Virus
49.	Bm IFV	*B. mori* Infectious FlacherieVirus
50.	Bm NPV	*B. mori* Nuclear Polyhedrosis Virus

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Chapter 1

General Introduction

STUDIES ON THE MICROSPORIDIA OF INSECT PESTS OF MULBERRY AND OTHER AGRICULTURAL CROPS

1.0 INTRODUCTION

Symbiosis refers to a close and prolonged interaction between organisms of different biological species. The term symbiosis means "living together" which is categorized into three different types of interactions *viz.* mutualism, commensalism and parasitism. Parasitism is a relationship between two species, where one gets benefitted, the parasite, at the expense of other species, the host. Parasites are organisms that evolved and adapted themselves to live on or within the hosts organisms; some have suffered dramatic reduction, as well as physical, genomic and functional adaptations. Parasites, common in most of the ecosystems, may become pathogenic and harm the host by causing tissue damage during their various life cycle stages. They may be categorized as ectoparasites if live on the external surfaces of their hosts, and endoparasites if they invade different tissues and organs of the host body. The parasitic way of life is generally so successful that it has evolved in almost every phylum of animals and plant groups, as well as in a diversity of species of bacteria, fungi, protozoa, helminthes, myxozoans and arthropods (Roberts and Janovy, 2005).

Insects comprise the largest group of organisms and are involved in various vital ecosystem services such as pollination, decomposition, herbivory and biological control, as well as contributing directly to human based economies through silk, lac insects and honey bee production. However, the insects are vulnerable to an assortment of diseases caused by microbes such as viruses, bacteria, fungi and the protozoans. Among these, microsporidia have been considered to be the most diversified intracellular fungal parasites that infect almost all invertebrates, specially insects, and cause a dreaded disease called microsporidiosis. Microsporidiosis of silkworm is caused by a highly virulent parasitic microsporidian, *Nosema bombycis*. The disease is very chronic to highly virulent and can result in serious losses to the sericulture industry. Microsporidia infect not only the silkworm, *Bombyx mori* L., but also different insect pests of mulberry and other agricultural crops. The infected insect pests of mulberry and also the insect pests from nearby agricultural crops that are casual visitors of mulberry gardens liberate the microsporidian spores along with their

scales and faecal matter on the mulberry leaf and thereby contaminate the mulberry leaf. As a result, different microsporidia gain entry into the silkworm rearing process through contaminated mulberry leaf and cross infect the silkworm. Keeping the above in view, the present study is a pioneering attempt to investigate the occurrence of microsporidia in the common insect pests of mulberry and other agricultural fields, to isolate, study and record their morphological characteristics; and to test the pathogenicity of the isolated microsporidia for the mulberry silkworm *B. mori* L.

1.1 MICROSPORIDIA

Microsporidia are a diverse group of obligate, eukaryotic, intracellular parasites that infect both invertebrate and vertebrate hosts including humans and have a worldwide distribution (Larsson, 1999; Sprague 1981, 1992, 1976, 1977; Weiss, 2001; Richards *et al.,* 2003). They are one of the most outstanding groups of organisms in many respects which were first identified 160 years ago with the description of *N. bombycis*, a pathogen of silkworm *B. mori* L. (Naegeli, 1857). The earliest record of this pathogen was seen in fish stickleback but the parasite was later identified as microsporidia and named as *Glugea anomala* by Moniez in 1887 (Gluge, 1838; Moniez, 1887). The description of *N. bombycis* by Nägeli is considered as the beginning of microsporidiology. The microsporidian parasites belong to the phylum Microspora (later Microsporidia) (Balbiani, 1882) and have been taxonomically problematic since their discovery (Keeling and Fast, 2002). Till date, about 200 genera and more than 1500 species of microsporidian parasites have been described (Vávra and Lukeš, 2013; Becnel and Andreadis, 2014). Among these, more than 200 species belong to the genus *Nosema* which is most prevalent in invertebrate hosts (Sprague, 1981). The microsporidium *N. bombycis* causes pebrine disease in silkworm which was responsible for the devastation of the European silk industry during the middle of the 19th century and continues to cause heavy economic losses in silk-producing countries such as China (Cai *et al*., 2011). Besides this, microsporidia also cause major economic losses in beekeeping with nosemosis (*Nosema apis* and *Nosema ceranae*) and in aquaculture with microsporidiosis (*Loma salmonae* for salmonids, *Thelohania* sp. for shrimps) (Becnel and Andreadis, 2001; Lom and Nilsen, 2003). A vast majority of microsporidian infections have been reported from metazoans where as some of the infection have also been described in the protists

such as ciliates, apicomplexans and also in bryozoans (Dykova and Lom, 1999; Fokin *et al*., 2008; Canning *et al.*, 2002). The microsporidia are also considered as natural intracellular parasites of the nematode *Caenorhabditis elegans* (Troemel *et al.*, 2008). They are also reported to infect the oligochaetes (Morris *et al*., 2005). Further, the microsporidian infections have been reported in a variety of domestic and wildlife mammalian hosts and also from avian, amphibian, and reptilian hosts (Snowden and Shadduck, 1999; Snowden and Phalen, 2004). The first microsporidia reported from mammals was *Encephalitozoon cuniculi*, which was found in the brain of laboratory rabbits (Wright and Craighead, 1922). In humans, the first case of microsporidian infection was reported in 1959 (Matsubayashi *et al*., 1959) and in course of time the microsporidian parasites emerged as opportunistic pathogens in AIDS patients. Several new genera and species have been discovered and described in humans worldwide (Shadduck, 1989; Shadduck and Greeley, 1989; Canning and Hollister, 1992; Bryan and Weber, 1993). Nowadays, these parasites are frequently recognized as etiologic agents of opportunistic infections in immunosuppressed and immunocompetent patients (Schwartz *et al.*, 1996, Keeling and McFadden 1998, Coyle *et al*., 2004, Didier, 2005; Lanternier *et al*., 2009, Talabani *et al*., 2010, Sak *et al*., 2011). Out of the 14 species of microsporidian parasites known to infect humans, the microsporidium *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are the most common causes of human infections and are associated with diarrhea and systemic disease (Didier, 2005). The fact that human-infecting microsporidia are also pathogens of several animal groups and are found in the environment as resistant spores suggests both zoonotic and environmental potential transmissions to humans. Due to the wide diversity of the microsporidian parasites as well as their evolutionary and medical significance, the research on Microsporidia has been intensified in a variety of disciplines.

1.1.1 History

The first microsporidian parasite was described in the 19th century when pebrine, or "pepper disease," ravaged the European silkworm industry, almost completely in France and Italy. From France the disease spread to Spain, Italy and Germany and, in 1860, the German silk industry was totally destroyed (Lebert, 1858). The pebrine agent was observed to be a microscopic parasite that was named *Nosema*

bombycis by Naegeli (1857). In the landmark studies '*Etudes sur la maladie des vers a soie'* ('Studies on the diseases of the silkworms), Pasteur (1870) described the methods for controlling and prevention of pebrine disease in silkworms. After another five years of research, Pasteur had observed that the parasite was found not only in the silkworm larva, but also in their moths and ova.

1.1.2 Taxonomy

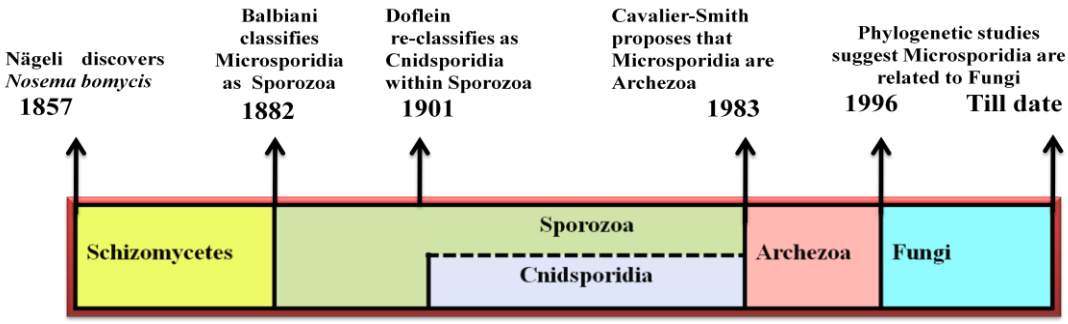


Figure 1: Timeline showing the changing taxonomic position of phylum Microsporidia, from their discovery in 1857 to the present. (Source: Keeling, 2009)

The phylum Microsporidia has always posed a difficult evolutionary problem since its discovery. Earlier they were thought to be ancient organisms that diverged from prokaryotes, but later studies suggested that microsporidia are true eukaryotes that underwent gene compaction and lost several genes as a result of their growing adaptation to intercellular parasitism (Edlind *et al*., 1996; Hirt *et al*., 1999; Keeling and Doolittle, 1996; Keeling *et al*., 2000; Keeling and Fast, 2002). They possess a typical eukaryotic nucleus, an endomembrane system, and a cytoskeleton (Mathis, 2000), but lack several eukaryotic features including mitochondria, peroxisomes, centrioles, stacked Golgi membranes, hydrogenosomes, glycosomes, nutrient storage granules and 80S ribosomes. They possess prokaryote like molecular and cytological characteristics such as 70S ribosomes, rRNAs (16S and 23S), 9 + 2 microtubule structures, compact and reduced genome and a fused 5.8S and 28S rRNA (Cavalier-Smith, 1998; Keeling and Fast, 2002; Texier *et al*., 2010). For the above reasons, the taxonomic designation of the microsporidia had been controversial for several years. Carl Wilhelm von Naegeli considered *Nosema* to be yeast like fungus and the member of the schizomycetes which, at that time, was a conglomeration of yeasts and bacteria (Pfeiffer, 1888). Balbiani (1882) first suggested the separate taxon Microsporidia

(Microsporidies) for *N. bombycis* recognizing that the parsite lacked characteristics of several Schizomycetes but shared similar characteristics with the Sporozoa (Leuckart, 1879). The microsporidia were most often believed to be a kin to Myxosporidia and Actinomyxidia and were included in the group Cnidosporidia, a position favoured for over 100 years (Kudo, 1918). Cavalier-Smith (1983) proposed that the microsporidia might be primitively amitochondriate eukaryotes as they lacked mitochondria and placed them in kingdom Archezoa, a group of organisms defined by their primitive lack of mitochondria. However, the recent molecular studies suggest that microsporidia shares a common origin with fungi (Fischer and Palmer, 2005) (Table 1).

1.1.3 Microsporidia- Fungi relationship

The evidence for the fungal origin of microsporidia has emerged from gene sequence analysis of beta and alpha tubulins (Edlind *et al*., 1996; Keeling and Doolittle, 1996). Later these findings were supported by the phylogenetic analysis of many protein coding genes *viz.* sequences of the DNA depended RNA polymerase II largest subunit (RPB1), the TATA-box binding protein (TBP), mitochondrial HSP70 (70 kDa heat shock protein), Valyl-tRNA synthases (ValRS), and seryl tRNA synthases (Germot *et al*., 1997; Hirt *et al*., 1997, 1999; Brown and Doolittle, 1999; Arisue *et al*., 2002; Williams *et al*., 2002).

Again the analysis of transcription initiation factor II (TF II), elongation factor EF-1α, vacuolar ATPase subunit A, GTP binding protein and the rRNA gene placed Microsporidia within or as a sister-group to Fungi (Van de Peer *et al*., 2000; Katinka *et al*., 2001; Fischer and Palmer, 2005). In fungal group, the microsporidia are closely related to zygomycetes (Keeling *et al*., 2000). Besides phylogenetic analysis, there are a number of common characters shared between Microsporidia and Fungi *viz.* formation of spores, chitinous spore wall, cryptomitosis, presence (in some species) of a formation of two adjacent nuclei called diplokaryon, some features of meiosis and the presence of trehalose (Flegel and Pasharawipas, 1995; Bigliardi and Sacchi, 2001; Cavalier-Smith, 2001; Thomarat *et al.*, 2004).

Further, the microsporidia possess a three component mRNA capping system similar to that of Fungi (Hausmann *et al*., 2002; Texier *et al.*, 2005).The SSU rRNA

gene of both Microsporidia and Fungi lack a paromomycin-binding site (Katiyar *et al.*, 1995). Again, the types of microsporidia septins, GTPases involved in organizing the sites of cell division, vesicle trafficking, apoptosis and cell movement support fungal affiliation to Microsporidia. Interestingly, the microsporidial septins are closely related to septins of yeast but not of other fungi (Pan *et al.*, 2007).

The most peculiar characteristic of Microsporidia is that it doesn't resemble either Fungi or sister group of Fungi in terms of structure whereas the molecular evidence supporting their relationship is overwhelming (Vávra and Lukeš, 2013). Altogether, evidence appears to be growing in support of a Microsporidia-Fungi relationship.

Table 1: A Comparison of Major Features of the Microsporidia and Fungi

Characteristics	**Microsporidia**	**Fungi**
Mitochondria	Absent	Present
Perixosomes	Absent	Present
Lysosomes	Absent	Present
Golgi Complex	Lack stacked cisternae	Variable, some stacked, some unstacked
Endoplasmic Reticulum	Smooth and rough	Smooth and rough
Ribosomes	Prokaryote-size (70S) consisting of large (23S) and small (16S) subunits; lack 5.8S but homolog found at beginning of 23S subunit	Typical eukaroytic size of 80S
Cytoskeleton	Microtubles, keratin filaments, probably actin and intermediate filaments	Microtubles, actin, and intermediate filaments
Centrioles	Absent, spindle attachment to "spindle plaque;" small "polar bodies" located near	Present only in Chytridiomycota (composed of nine triplets), replaced by spindle pole bodies

	the spindle plaque	(SPBs) in most true fungi. Multivesicular bodies associated with the SPB
Chitin	Present in endospore	Present in cell and spore wall
Resting stage (spore)	Polymorphic, uninucleate, and binucleate spores. Some species produce only one type of spore, some produce as many as four types	Sexually (oospores, zygospores, ascospores, basidiospores) and asexually (conidia or sporangiospores) are produced, all uninucleate
Vegetative growth	Binary fission and multiple fission	Walled hyphae develop by apical growth, in yeast by budding or binary fission
Habitat	Obligatory parasites	Facultative and obligatory parasites
Hosts	From protists to humans	Plants, animals, and other fungi

1.2 GENERAL FEATURES OF MICROSPORIDIA

1.2.1 Mature spore

Microsporidia are highly reduced eukaryotes at every level, from morphology and ultrastructure, to biochemistry and metabolism, and even at the molecular level (Keeling and Fast, 2002). They survive only in the form of tiny spores outside the host cell i.e. they survive in the open air, water and soil and are transmitted to the new host through contamination (Franzen and Müller, 1999; Vávra and Larsson, 1999). The size and shape of spores is highly varied among the species with differences in the number of nuclei, thickness of exospore and endospore walls, and structural differences within the species (Dunn and Smith, 2001). The size of the spore varies from 1-40 µm in length to 1.5-5 µm in width. For example, spore size as little as 1 µm in *E. bieneusi* to 40 µm in *Bacillidium filiferum* has been reported (Vávra and Larsson, 1999). The spore size of *Nosema* sp. ranges from 3-7 µm in length and 1.5-4.5 µm in width (Choi *et al*., 2002; Bashir and Sharma, 2008; Sharma *et al*., 2014; Abdel-Baki *et al*., 2016). Again, the shape of the spore may be oval, round, pyriform or rod-like depending on the species. The spores are able to withstand high internal

pressure (>7.0 Mpa) generated inside the spore during germination (Lom and Vavra, 1963).

The spore is bounded by a thick cell wall consisting of three layers: the external electron-dense and proteinaceous exospore layer, the middle electron-lucent endospore layer which is composed of a complex of proteins and α-chitin, and an inner highly electron dense membrane, the plasmalemma (Kudo, 1921; Canning and Lom, 1986). The cell wall provides mechanical resistance to the spore from environmental influences. Further, the endospore layer is less thick and more electron-dense at the anterior end of the spore. The matrix of the spore, called sporoplasm, is enclosed by the plasma membrane and possesses one or two nuclei, ribosomes, a posterior vacuole and a hollow coiled polar filament (Undeen, 1997). The polaroplast is an organization of membranes occupying the anterior part of the spore. The anterior portion of the polaroplast exists as highly organized, closely stacked membranes called the lamellar polaroplast, whereas the posterior portion is loosely organized and consists of several widened or inflated vesicle-like cisternae called as vesicular polaroplast (Figure 1.1).

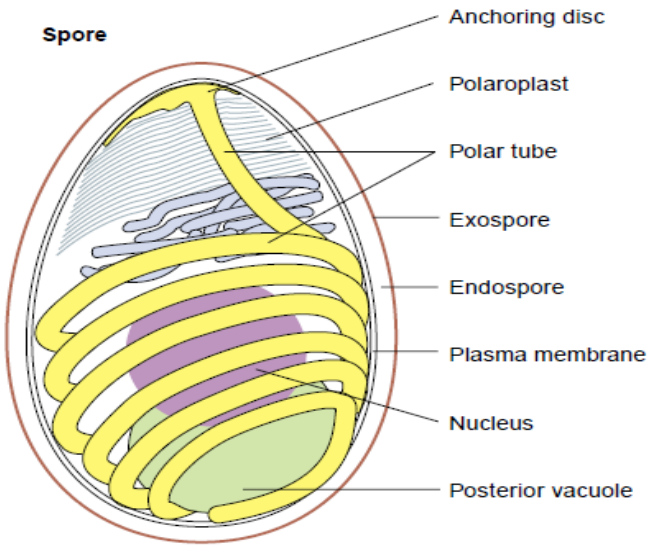


Figure 1.1: Diagram of a microsporidian spore showing the major structures. (Source: Franzen, 2004)

1.2.2 Polar tube: the invasive organelle of microsporidia

The extrusion apparatus of the spore is called polar tube or polar filament and is attached to the inside of the anterior end of the spore via an umbrella-shaped

structure, the anchoring disk, from which it extends to the posterior of the spore (Wittner and Weiss, 1999; Levine *et al*., 1980). The polar tube is divided into two regions: the anterior straight portion surrounded by a lamellar polaroplast and the posterior coiled region that forms 4-30 coils around the sporoplasm, depending on the species (Huger, 1960, Vávra *et al*., 1966; Cali and Owen, 1988). The polar tube is composed of membrane and glycoprotein layers and ranges from 50-500 μm in length and 0.1-0.2 μm in diameter (Frixione *et al*., 1992; Keohane and Weiss, 1999; Vavra and Larsson, 1999).

The polar tube is a unique organelle which is everted rapidly from the anterior pole of the spore during infection and penetrates the host membrane like a needle. Subsequently, the sporoplasm is discharged into the host cytoplasm through the polar tube (Keohane and Weiss, 1998; Bigliardi and Sacchi, 2001; Franzen, 2004; Xu and Weiss, 2005; Delbac and Polonais, 2008) (Figure 1.2). The polar tube has some flexibility in that it shows variation in diameter from 0.10-0.25 μm during discharge (Scarborough- Bull and Weidner, 1985), its diameter can increase to 0.4 μm during sporoplasm passage (Lom and Vavra, 1963, Ishihara, 1967, Weidner, 1972, 1982), and its length shortens by 5-10% after sporoplasm passage (Frixione *et al*., 1992). Furthermore, the number of polar tube coils, their arrangement, and the angle of helical tilt are conserved and constitute the identifying characteristics for the respective microsporidian species (Sprague *et al*., 1992; Vávra and Larsson, 1999).

1.2.2.1 External stimuli for polar tube discharge from spore

The spore discharge is believed to occur by following several steps (Keohane and Weiss, 1998):

i. Activation by appropriate stimuli
ii. Increase in the intrasporal osmotic pressure
iii. Eversion of the polar tube
iv. Passage of sporoplasm through the polar tube

The external stimuli that have been shown to promote spore discharge include incubation at an alkaline pH (Undeen and Epsky, 1990), acidic pH (Undeen, 1978, 1983), or a pH shift from acid to alkaline (Pleshinger and Weidner, 1985) or from alkaline to less alkaline or neutral (Undeen, 1978; Malone, 1984). Some species have

demonstrated spore discharge at both acidic and alkaline conditions (Hashimoto *et al*., 1976; Undeen, 1983; Undeen and Avery, 1988a). Dehydration followed by rehydration has been effective in promoting spore discharge in some species (Whitlock and Johnson, 1990). Various cations including potassium, lithium, sodium, cesium (Ishihara, 1967; Undeen, 1978; Malone, 1990; Whitlock and Johnson, 1990; De Graaf *et al*., 1993; Frixione *et al*., 1994) and anions such as bromide, chloride, iodide and fluoride (Undeen and Avery, 1988a) have been used to promote discharge. It is apparent from these studies that both cations and anions enter the spore passively. Mucin or polyanions (Weidner *et al*., 1995), hydrogen peroxide (Kudo, 1918; Lom and Vávra, 1963; Hashimoto *et al*., 1976; Leitch *et al*., 1993), low dose ultraviolet radiation (Undeen and Vander, 1990), and Na^+ ionophore monensin (Frixione *et al*., 1994) and calcium ionophore A 23187 (Weidner and Byrd, 1982) have also been used to trigger discharge. While the spore wall forms a barrier to larger molecules (i.e. it functions as a molecular sieve), it appears that alkali metal cations freely pass through the spore wall and plasma membrane of the spore. These cations are required for the germination process and that, to some extent, the smaller cations are more effective (Frixione *et al*., 1994).

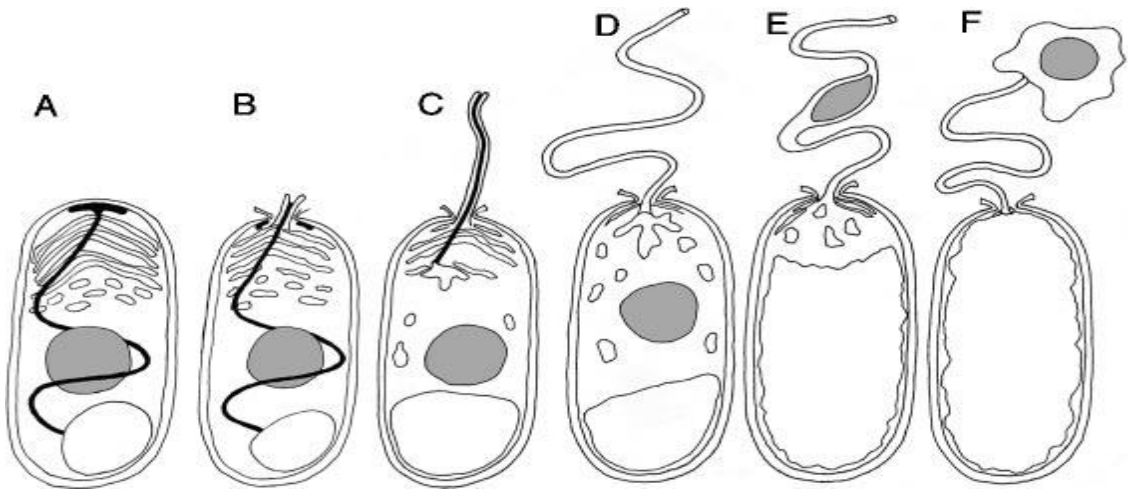


Figure 1.2: Polar tube eversion during spore germination. (A) Dormant spore, showing polar filament (black), nucleus (gray), polaroplast and posterior vacuole. (B) Polaroplast and posterior vacuole swelling, anchoring disk ruptures, and polar filament begins to emerge, everting as it does so. (C) Polar filament continues to evert. (D) Once the polar tube is fully everted, the sporoplasm is forced into and (E) through the polar tube. (F) Sporoplasm emerges from the polar tube bound by new membrane. (Source: Keeling and Fast, 2002)

1.2.2.2 Inhibitors of polar tube discharge from spore

Inhibitors of spore discharge include 0.01-0.1 M magnesium chloride (Malone, 1984), ammonium chloride (Undeen, 1978; Undeen and Avery, 1988; Undeen and Epsky, 1990), low salt concentrations (10-50 mM) (Undeen, 1978), sodium fluoride (Undeen and Avery, 1988), silver ions (Ishihara, 1967), gamma radiation (Undeen *et al*., 1984), ultraviolet light (Whitlock and Johnson, 1990), temperatures greater than 40°C (Whitlock and Johnson, 1990), calcium channel antagonists (lanthanum, verapamil, nefedipine), calmodulin inhibitors (chlorpromazine, trifluroperazine) (Pleshinger and Weidner, 1985; Leitch *et al*., 1993), a microfilament disrupter (cytochalasin D). Calcium chloride (0.001 to 0.1M) has been found to inhibit spore discharge in some studies (Oshima, 1964; Ishihara, 1967; Undeen, 1978; Weidner and Byrd, 1982; Malone, 1984), while 0.2 M $CaCl_2$ at pH 9.0 (Pleshinger and Weidner, 1985) and 1 mM $CaCl_2$ (Leitch *et al*., 1993) promoted discharge in other studies. Removal of clathrin and calmodulin from the intermediate filament cage assembly which envelopes the spores of *Spraguea lophii*, resulted in irreversible inactivation of spore discharge (Weidner, 1992).

1.2.3 Mechanism behind polar tube extrusion and invasion into host cell

Polar tube extrusion was first reported by Thelohan (1892, 1894) and subsequently confirmed by others (Stempel, 1909; Korke, 1916; Kudo, 1918). The polar tube discharges an explosive reaction occurring in less than 2 seconds, with a mean acceleration and velocity of about 500 $\mu m/sec^{-2}$ (Oshima, 1937; Lom and Vávra, 1963; Weidner, 1972; Frixione *et al*., 1992).

The signaling pathways that lead to polar tube extrusion *in vivo* remain largely unknown. However, experimental evidences suggested an increase in osmotic pressure inside the spore followed by a rapid influx of water through the cell membrane (Keohane and Weiss, 1998, 1999; Williams, 2009). Ghosh *et al*. (2006) postulated intra-membranous aquaporin-like channels to be the mechanism for the influx of water into the activated spore which resulted in the swelling of both the lamellar polaroplast and the posterior vacuole and then in polar tube extrusion (Figure 1.2, 1.3 and Figure 1.4). Polar tubes have been observed to be rapidly digested after

extrusion in digestive fluid or in the midgut of insects (Oshima, 1937; Undeen, 1976; Undeen and Epsky, 1990).

The mechanism behind the penetration of host cell by polar tube is still unclear; however a recent study using *Encephalitozoon* sp. suggested that the polar tube entered new host cells by a phagocytic process which was visible under scanning electron micrographs (Schottelius *et al*., 2000). Some components of the polar tube and the spore wall have been suggested to be involved in tissue recognition and thereby in the initiation of host cell invasion. Microsporidian spores are believed to utilize glycosaminoglycans (GAGs) as host cell receptors for cell adhesion and infection (Hayman *et al*., 2005), through the possible interaction with a major spore wall protein named EnP1 (Southern *et al*., 2007). Moreover, the O-mannosylated moiety of PTP1 can interact with a host cell mannose receptor which is the initial step leading to polar tube extrusion and cell invasion however, detailed characterization of spore wall protein are still needed (Xu and Weiss, 2005).

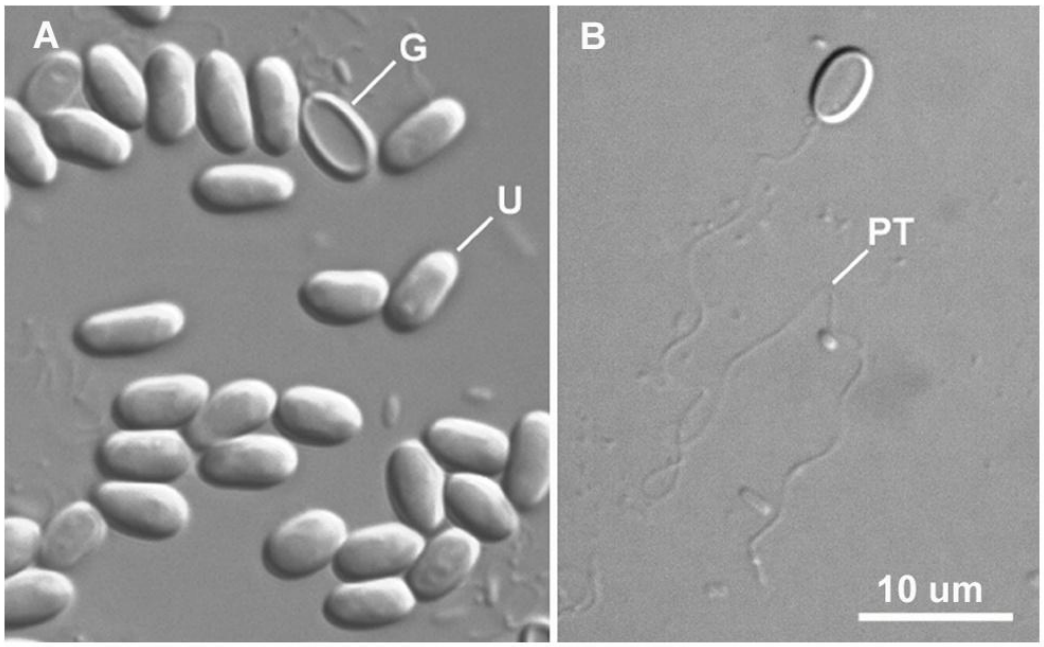


Figure 1.3: Light micrograph of *Antonospora locustae* with pressure-induced polar tube eversion. The scale bar is 10 um. (A) Many ungerminated spores (one example labeled U) and a few germinated spores, showing the residual spore wall (one example labeled G). (B) A germinated spore where the everted polar tube (PT) has extended far from the cell and can be seen to be many times the length of the spore (Source: Keeling, 2009).

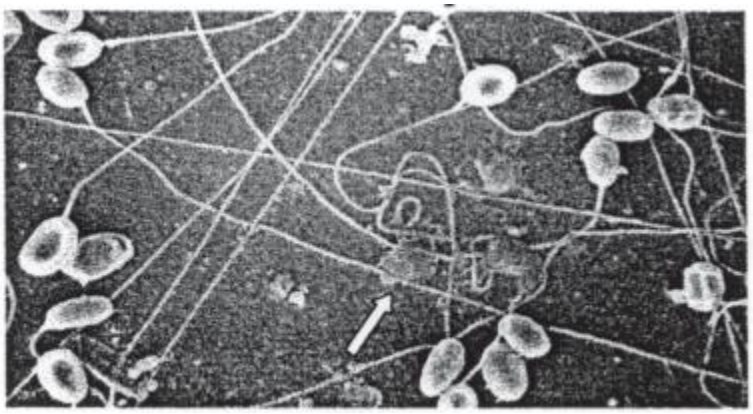


Figure 1.4: Germinated microsporidian spores with extruded polar tube (Source: Cali *et al.*, 2002).

1.3 LIFE CYCLE OF MICROSPORIDIA

For most microsporidians, the development of the parasite starts immediately after the sporoplasm enters into the host cell through the piercing polar tube. The development of parasite inside the host cell is variable depending on the family and/or genus of the microsporidium (Fig. 1.6). Most microsporidia infect the host cell cytoplasm; however, the genera *Nucleospora* (Docker *et al*., 1997), *Enterospora* (Stentiford *et al.*, 2007), and *Desmozoon* (Freeman and Sommerville, 2009) infect the nucleoplasm of its host cells, while some may develop in both the nucleus and cytoplasm (Palenzuela *et al*., 2014; Stentiford *et al*., 2007).

The life cycle of microsporidia can be divided into three phases (Fig 1.5)

1) Environmental/Infective phase
2) Proliferative phase (Merogony)
3) Sporogonic phase (Sporogony)

1.3.1 Environmental/Infective phase

The infective phase of microsporidia includes the liberation of spores into the environment and the spores are the only stages which exist outside the host cell. This phase can be divided into two parts *viz.* the factors which induce spore survival in the extracellular environment and the factors that activate the spore for germination (Cali and Takvorian, 2014).

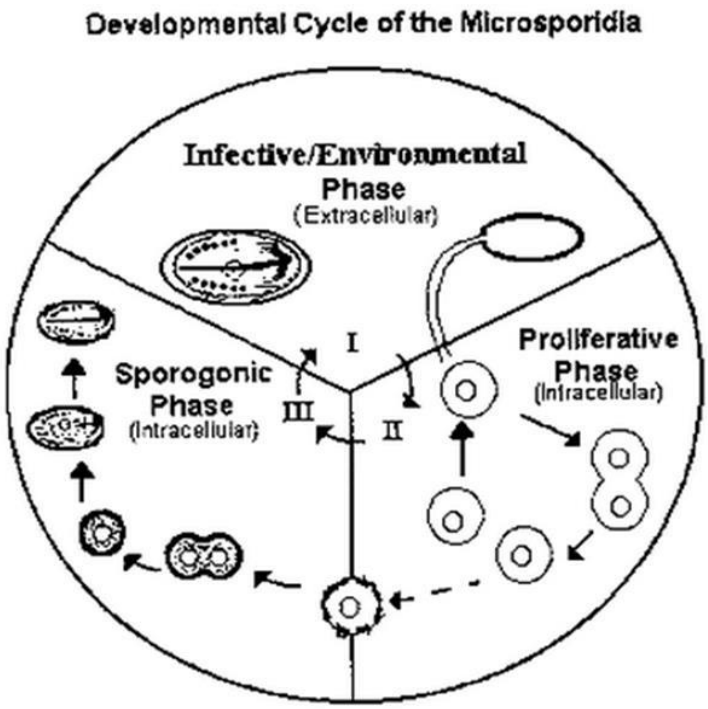


Figure 1.5: Diagram of a typical developmental cycle of the Microsporidia

The three regions represent the three phases of the microsporidian life cycle. Phase I is the infective/environmental phase, the extracellular phase of the cycle. It contains the mature spores in the environment. Under appropriate conditions the spore is activated (e.g., if the spore is ingested by an appropriate host, it is activated by the gut environment) and triggered to extrude its polar filament (which becomes a hollow tubule). If the polar tubule pierces a susceptible host cell and injects the sporoplasm into it, phase II begins. Phase II is the proliferative phase, the first phase of intracellular development. During the proliferative part of the microsporidian life cycle, organisms are usually in direct contact with the host cell cytoplasm and increase in number. The transition to phase III, the sporogonic phase, represents the organism's commitment to spore formation. In many life cycles this stage is indicated morphologically by parasite secretions through the plasma lemma producing the thickened membrane. The number of cell divisions that follow varies depending on the genus in question, and the result is spore production. (Source: Cali and Takvorian, 1999).

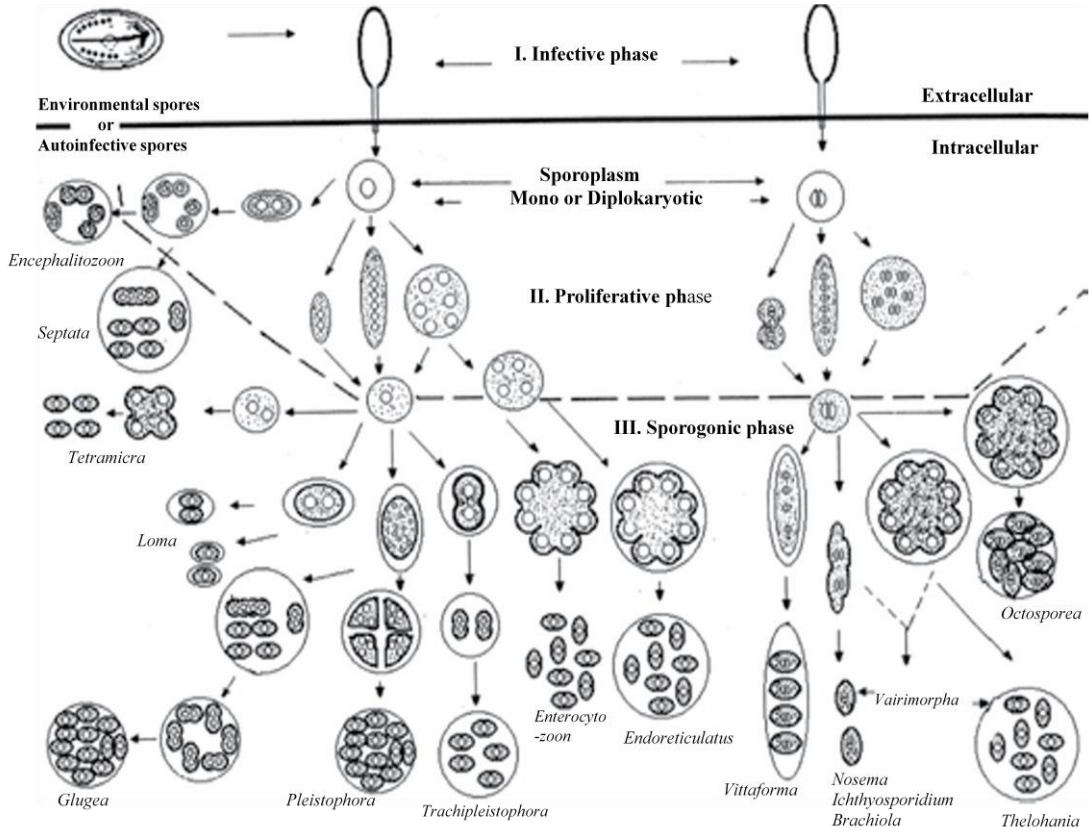


Figure 1.6: Diagrammatic representation of the life cycles of several horizontally transmitted genera of the microsporidia, illustrating both the three-part life cycle common to these organisms and some of the diversity of their development within the proliferative and sporogonic phases (Source: Cali and Takvorian, 2014)

1.3.2 Proliferative phase (merogony)

The proliferative phase (merogony) begins immediately after the entrance of sporoplasm into a suitable host cell. The proliferative cells, referred to as meronts, are round in shape and encircled by a typical unit membrane.

The meront stages of microsporidia develop in direct contact with the host cell cytoplasm (*Nosema*, *Enterocytozoon*), within a parasitophorous vacuole lined by a host-produced single membrane (*Encephalitozoon*), in a parasite-secreted amorphous coat (*Pleistophora*, *Trachipleistophora*, *Thelohania*), surrounded by the host endoplasmic reticulum (*Endoreticulatus, Vittaforma*) or within a xenome (Martinez *et al.*, 1993; Sprague *et al.*, 1992; Frixione *et al.*, 1997; Bigliardi and Sacchi, 2001; Cali and Takvorian, 2014). The xenome protects the parasite from the host immune response and confines it to one site, preventing its dissemination throughout the host

body (Sprague and Hussey, 1980). Xenomas have been found in oligochaetes (e.g., *Bacillidium, Burkea, Hrabyeia, Jirovecia*), crustaceans (e.g., *Abelspora, Mrazekia*), insects (e.g., *Polydispyrenia, Thelohania*) and poikilothermic vertebrates, mostly fish (*Alloglugea, Amazonspora, Glugea, Ichthyosporidium, Loma, Microfilum, Microgemma, Neonosemoides, Pseudoloma, Spraguea, Tetramicra*) (Lom and Dyková, 2005).

Meronts have a large nuclear region which is either monokaryotic with a single nucleus or diplokaryotic with two nuclei, depending on the species. The cytoplasm contains a few elements of rough endoplasmic reticulum (RER) and abundant ribosomes free in the cytoplasm (Morrison and Sprague, 1981).

Actin has been detected in meronts by transmission electron microscopy, fluorescence microscopy and immune-electron microscopy (Bigliardi *et al.*, 1999). The meronts undergo repeated division by binary fission (*Encephalitozoon, Nosema, Vittaforma*), or karyokinesis occurs without cytokinesis, producing multinucleate cells called merogonial plasmodia (*Enterocytozoon, Pleistophora, Trachipleistophora*) **(**Cali and Takvorian, 2014).

1.3.3 Sporogonic phase (Sporogony)

Sporogony involes the development of a meront into a sporont which produces sporoblast. The sporoblast then undergo maturation and transforms into a mature spore without any further division (Hossain *et al.*, 2012). The sporont may divide in distinct ways but the mechanism of the mitotic process is the same as for meronts. If the karyokinesis is linked to the cytokinesis, sporonts divide by binary fission and two sporoblasts are formed and this type of division occurs in genera *Nosema, Encephalitozoon, Vittaforma*. In the other case, cytokinesis is not linked to karyokinesis and multinucleate sporonts develop within a sporogonial plasmodium as in the case of *Enterocytozoon* and *Pleistophora* (Bigliardi and Sacchi, 2001). Maturation of the sporoblasts occurs by thickening of the membrane to form a electron dense surface, the exospores (Cali and Takvorian, 1999, Lom *et al.*, 2000). It is also characterized by the progressive increase in cytoplasmic density along with the development of ribosomes, smooth and rough ER, enlargement of larger Golgi complexes, as well as development of the extrusion apparatus (Bigliardi and Sacchi,

2001). Sporogony may or may not occur within a membranous envelope that originates from either from the host or from the parasite or from both, and is designated as a sporophorous vesicle (Cali and Takvorian, 1999). The size of mature spore is smaller than its developmental stages and the mature spores liberate when the host cell disintegrates. Fresh microsporidian spores are extremely refractile when viewed by phase contrast microscopy.

1.4 TRANSMISSION OF MICROSPORIDIA

Transmission of any pathogen plays a key role in the host-parasite interaction which can ultimately influence the population dynamics of the host. Out of the 200 described genera of microsporidia, about 93 genera have been reported from the insect host (Becnel and Andreadis, 2014). Most of the microsporidian species are transmitted horizontally (from secondary sources) to a new hosts, while some are transmitted vertically (female-to-offspring transmission) or some of the microsporidia transmitted by a combination of the two modes (Dunn and Smith, 2001; Dunn *et al.*, 2001). The major and minor pathways through which the microsporidia are transmitted into insect hosts are presented in Figure 1.7.

1.4.1 Horizontal Transmission

The invertebrate animals are most susceptible to horizontally infectious microsporidian spores when they are actively feeding and also during their immature developmental stages (Solter, 2014). The most common method of horizontal transmission is through direct oral ingestion of infectious spores found in food or liquids within the insect's immediate environment (Canning, 1971; Andreadis, 1987; Campbell *et al.*, 2007; Hoch *et al.*, 2008; Goertz and Hoch, 2011). However, the transmission is dependent on the acquisition of a sufficient quantity of spores as well as appropriate conditions (pH, enzymes) within the host's alimentary tract to facilitate spore germination and successful entry into susceptible host tissues (Tanada, 1976). Contamination of the environment with spores is typically achieved when an infected host dies or when spores are released in fecal excrement (Goertz *et al.*, 2007; Goertz and Hoch, 2008a). In general, spores of enteric microsporidia that infect the alimentary tract are almost always disseminated in faeces (Weiser, 1961). Pathogen dissemination and subsequent oral transmission may also be effected through

regurgitation of spores and through the secretion of spores in larval silk where these glands are heavily infected. The former has been reported with *Nosema fumiferana* in the spruce budworm (*Choristoneura fumiferana*) (Thomson, 1958; Wilson, 1982) and the latter with an undescribed *Nosema* sp. in the gypsy moth (*Lymantria dispar*) (Jeffords *et al.*, 1987; Goertz *et al.*, 2007).

Direct insect-to-insect transmission via oral ingestion of microsporidian spores occurs through cannibalistic feeding on weak or moribund infected individuals or by feeding on infected cadavers (Brooks, 1988). According to Kramer (1976), cannibalism probably occurs to some extent in all gregarious insect hosts for microsporidia. It is reported to be the primary method of horizontal transmission of *Nosema locustae* in rangeland grasshopper populations (Henry, 1972) and has been observed in Indian meal moth larvae *Plodia interpunctella* (Lepidoptera) infected with *Vairimorpha plodiae* (Kellen & Lindegren, 1971) and in flour beetles *Tribolium confusum* and *Tribolium castaneum* (Coleoptera) infected with *Nosema whitei* (Watson, 1979).

Many species of microsporidia found to infect humans also infect a wide range of animals supporting the likelihood that zoonotic transmission occurs (Dengjel *et al.*, 2001). The microsporidia transmitted to human by various sources *viz.* through contaminated water (ground, surface and ditch water), food (Slifko *et al.*, 2000; Orlandi *et al.*, 2002; Dascomb *et al.*, 2000) (Figure 1.8).

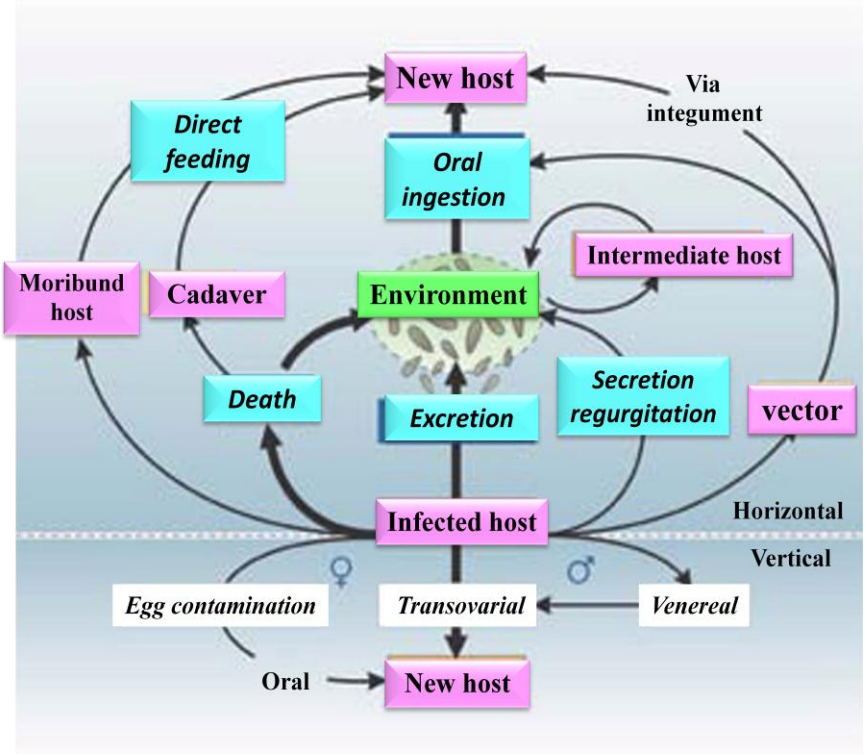


Figure 1.7: Diagram of the major and minor pathways of transmission for insect-parasitic microsporidia (Source: Becnel and Andreadis, 2014)

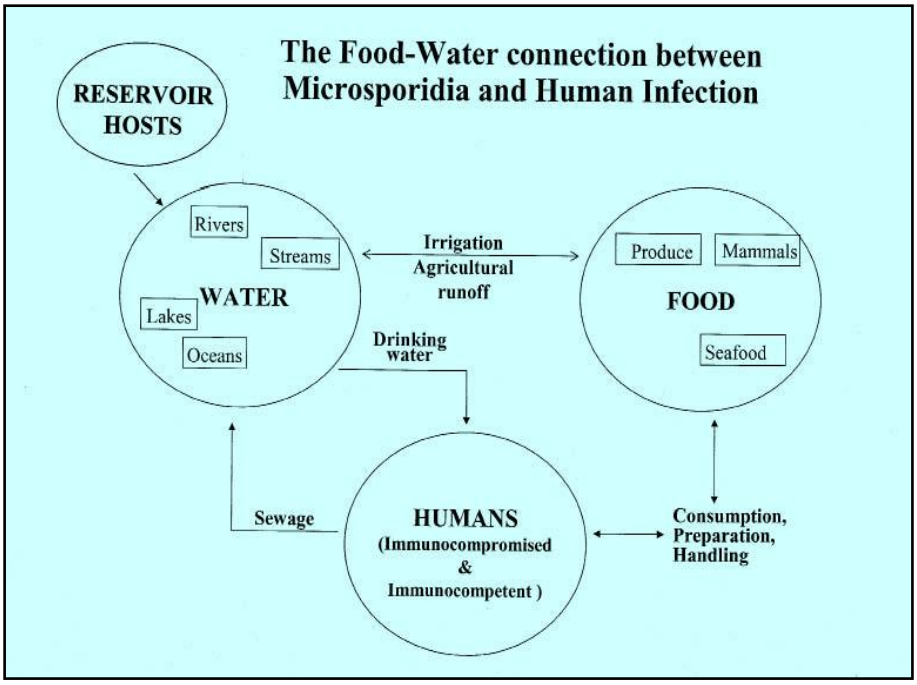


Figure 1.8: The food-water connection between microsporidia and human infection (Source: Cali *et al.*, 2004).

1.4.2 Vertical transmission

The transmission of microsporidia from an infected female to its offspring is called vertical transmission. Such transmission takes place within a host lineage and the parasites transmitted vertically from one generation to the next generation of hosts (Dunn *et al.,* 2001). Transplacental transmission has been reported for microsporidia which infect vertebrates *viz.* rodents, rabbits, carnivores, and non-human primates (Didier *et al.*, 1998; Snowden and Shadduck, 1999), whilst transovarial transmission is common in microsporidia infecting invertebrates (Dunn *et al.*, 2001). Vertical transmission of microsporidiosis in humans has not been reported. Therefore, the vertical transmission of microsporidia is mainly uniparental (maternal) because only the female hosts transmit the parasites either transplacentally or by transovarially from mother to zygote via the cytoplasm of the egg. Male gametes are extremely small and rarely contribute to the cytoplasm inherited by the zygote (Andreadis, 2007; Szeredi *et al.,* 2007; Baneux and Pognan, 2003). Further, in invertebrate host, this uniparental inheritance imposes unusual selection pressures on host–parasite coevolution which have led to the evolution of two unusual traits in the microsporidia; and feminisation of the host (Terry *et al.*, 1998).

1.5 SIGNIFICANCE OF THE STUDY

India has a unique distinction of being the only country in the world producing four varieties of silk, and produced 20,478 MT of mulberry, 2819 MT of tasar, 5060 MT of eri and 166 MT of muga silk for the session 2015-16 (Central Silk Board, Annual Report, 2016). Among these, the common mulberry silkworm *B. mori* L. (Lepidoptera: Bombycidae) spins valuable silk fibre, making it one of the most beneficial insects to mankind, and is becoming an attractive multifunctional material for both textile and non-textile uses. Almost all commercial silk is made from cocoons spun by silkworms of the genus *Bombyx*. All silkworms suffer from various diseases like Pebrine (Protozoa: Microsporidia), Flacherie (Bacteria), Grasserie (Virus) and Muscardine (Fungus). These pathogens have a great role in deterioration of sericulture industry.

Members belonging to the genus *Nosema* are the most common microsporidia infecting Lepidoptera (Tsai *et al*., 2003). They are also responsible for causing the

most deadly diseases in silkworms; called microsporidiosis. They are also responsible for infectious diseases in humans and considerable problems in fisheries and sericulture industries. Thus, the absence or presence of these parasites determines the success or failure of the silk industry of a country. (Rao *et al*, 2004). Further, the insect pests of mulberry may act as natural reservoirs of Microspora which are cross-infective to silkworms. Several studies have reported the incidence of microsporidian parasites, as well as their cross infectivity to the silkworm (Bhat and Nataraju, 2004; Singh *et al.*, 2008; Bashir and Sharma, 2008). Such microsporidia, therefore, constitute a potential threat of gaining access to silkworm rearing through contaminated mulberry leaf and perpetuate the infection despite routine care taken in mother moth examination and sanitation. This may have an adverse impact on the sericulture industry.

1.6 OBJEVTIVES OF THE STUDY

The principal objective of the present investigation is to investigate the occurence of microsporidian infection in the insect pests of mulberry and other agricultural crops, to isolate these microsporidia for morphological and ultrastructural characterization and identification, and to investigate the infectivity of the isolated microsporidia to the silkworm *Bombyx mori* L. The present study was taken up with the following objectives:

1. Collection and screening of insect pests of mulberry and other agricultural crops for microsporidian infection.
2. Isolation and identification of microsporidian spores from the collected insect pests.
3. Morphological studies on the microsporidian spores isolated from insect pests of mulberry and agricultural crops by light microscopy
4. Ultrastructural study of the isolated microsporidian spores of the collected insect pests by Scanning Electron Microscopy (SEM).
5. Studies on the infectivity of the microsporidian spores isolated from insect pests of mulberry and agricultural crops to the silkworm *Bombyx mori* L.

The work embodied in this thesis has been organized into the following chapters:

Chapter 1: General introduction

Chapter 2: Review of literature

Chapter 3: Prevalence of microsporidian infection in the insect pests collected from mulberry field and other agricultural crops

Chapter 4: Microscopic study of microsporidian spores isolated from insect pests of mulberry and other agricultural crops

Chapter 5: Studies on the infectivity of the microsporidian spores isolated from insect pests of mulberry and other agricultural crops to the silkworm *Bombyx mori* L.

Chapter 2

Review of Literature

REVIEW OF LITERATURE

Microsporidia is the most diverse entomopathogens which has a broad host range but highly prevalent in fish and insects. Till date, approximately 200 genera of microsporidia have been described and yet many to be reported (Becnel and Andreadis, 2014).

2.1 INCIDENCE AND PREVALENCE OF MICROSPORIDIAN INFECTION IN INSECTS

Chen *et al.* (2008) have conducted a study on the presence *N. ceranae* infection in the European honey bee (*A. mellifera*) in the United States. They have collected honey bee samples between the year 1995 and 2007 from 12 states and examined them for the presence of *Nosema* infections. Among180 bees examined for *Nosema*, 16% of the bees (28/180) were found to be positive with *N. ceranae* infection.

Antunez *et al.* (2009) worked on the immune suppression in the honey bee *Apis mellifera* following infection by *N. ceranae* (microsporidia). They observed that, *N. ceranae* infection significantly suppresses the honey bee immune response, however this effect was not observed following the infection with *N. apis*. They concluded the immune suppression also increases the susceptibility to other bee pathogens and senescence.

Chen *et al.* (2009a) have conducted study on Asymmetrical coexistence of *N. ceranae* and *N. apis* in honey bees. The result showed that, *A. cerana* was infected not only with *N. ceranae* as previously reported, but also with *N. apis*. Both microsporidia produced single and mixed infections.

Dong *et al.* (2010) isolated five microsporidia including *Nosema heliothidis* and four others microsporidia from insects *viz. Pieris rapae, Phyllobrotica armta, Hemerophila atrilineata,* and *Bombyx mori* respectively and constructed two phylogenetic trees by sequencing their complete small subunit rRNA (SSU rRNA). They determined the taxonomic status of four novel microsporidia by analysising their phylogenetic relationship, G+C content, identity and divergence of the SSU

rRNA sequences. The data showed the microsporidia isolated from *Pieris rapae*, *Phyllobrotica armta*, and *Hemerophila atrilineata* had a close phylogenetic relationship with the genus *Nosema*, while the microsporidium isolated from *Bombyx mori* was closely related to the *Endoreticulatus*.

Chaimanee *et al.* (2010) have conducted study on Infections of *N. ceranae* in four different honeybee species. The microsporidium *N. ceranae* is detected in honeybees in Thailand for the first time. From 80 *A. mellifera* samples, 62 (77.5%) were positively identified for the presence of the *N. ceranae*.

Gajger *et al.* (2010) collected a total of 204 dead honeybee samples from different localities in and around Croatia and investigated them for the prevalence of *Nosema ceranae* infection. They studied the parasites by light microscopic examination and multiplex PCR. The microsporidian infections were found in honeybee samples collected from 21 districts in all the varied climatic conditions.

Bashir *et al.* (2011) have screened the insect pests of mulberry and agricultural crops for the presence of microsporidian infection and have found positive infection in *Pieris rapae, Catopsilia crocale, Catopsilia pyranthe, Spilosoma oblique* and *Daphnia pulverulentalis*.

Kyei-Poku *et al.* (2011) collected bronze birch borer *Agrilus anxius* Gor (Coleoptera: Buprestidae) near Sudbury and Sault Ste Marie, Canada and isolated microsporidia from the pest. They studied the prevalence, morphological characteristics both by light and electon microscopy of the microsporidium. Phylogenetic analysis confirmed that the microsporidian species from *A. anxius* is most closely related to the genus *Cystosporogenes* clade of microsporidia. More than 80% of the beetles were found infected in Sudbury and Sault Ste Marie beetle populations which was relatively stable in 2006-2007 but declined in 2008.

Botias *et al.* (2012) carried out a research work on the *Nosema* sp. infection in the honey bees (*Apis mellifera iberiensis*) colony and their negative effects. They characterize the sub-clinical and clinical signs of *N. ceranae* infection on honey bee colony strength and productivity and evaluated that the evolution of 50 honey bee colonies naturally infected by *Nosema* over a period of one year. Under experimental

conditions they found, *N. ceranae* infection was highly pathogenic for honey bee colonies, producing significant reductions in colony size, brood rearing and honey production.

Bollan *et al.* (2013) collected 71 honeybee *(A. mellifera)* colonies from Scottish Beekeeper's Association members screened for the presence of microsporidian infection, among which only 11 colonies were found positive with infection. They confirmed the presence of microsporidian parasites, *N. ceranae* and *Nosema* in 70.4% colonies by PCR analysis. Among the infected colonies, 70.4% were infected by both the pathogens with only 4.2 or 7% having either strain alone and 18.3% being *Nosema* free. The presence of *N. ceranae* was confirmed by gene sequencing method which indicated as a new variant. Differential quantification of the PCR product revealed, *N. ceranae* to be the widespread pathogen in Scotland that exists in combination with the endemic *N. apis*.

2.2 STUDY OF MICROSPORIDIA BY MICROSCOPY (LIGHT MICROSCOPY, SEM, TEM)

Liu (1973) described the detailed Structure of microsporidium *Nosema apis* Zander. They discussed the role of endoplasmic reticulum in the formation of polar filament, polaroplast and spore coat. Their study revealed that the mature spore was covered by a thick spore coat and a particle-bearing spore membrane. Polar filament was arranged in two layers and observed spirally running towards the posterior pole of the spore.

Odindo and Jura (1992) described the Ultrastructure and development of a microsporidium *Nosema marucae* isolated from the legume pod borer *Maruca testulalis*. The meronts were binucleated with an irregular cell body whereas sporonts were having many rough endoplasmic reticulums. Granular cytoplasm was found in the sporoblasts with a large number of ribosomes; cellular cleavage and nuclear division were also prevalent in the sporoblast. The ultrastructure of the mature spores showed a rough exocuticle with sculptured surface, a thinned endocuticle with polar tube of 12-15 coils. At the point of attachment, the anchoring disc was thick. The polaroplast was multilayered with two distinct regions.

Streett and Henry (1993) investigated the ultrastructure of *Nosema acridophagus* Henry isolated from the fat body of a grasshopper *Melanoplus sanguinipes*. Diplokaryotic stages were observed throughout the life cycle and developed in direct contact with the host cytoplasm. Plasmalemma of the meront was covered by a layer of tubular elements that transversely encircled the parasite. Polar tube was isolfilar with 10-12 coils arranged in a single row and the coil diameter ranged from 75-105 nm.

Cheung and Wang (1995) studied the ultrastructure of mirosporidium *Nosema mesnili* isolated from the Malpighian tubules of lepidopteran butterfly *Pieris canidia*. They also described various stages of life cycle *viz*. meronts, sporonts, sporoblasts, and spores, with typical diplokaryon in each stage. Meronts measured 3.8±0.2 µm in diameter whereas mature spores was oval in shape, measured 1.5-2.0 µm in width and 3.0-4.0 µm in length with polar filament of 11 coils.. They determined angle of tilt of the coils to the long axis which was approximately 10-15º.

Becnel *et al.* (2002) reported a new microsporidian species from the predatory mite, *Metaseiulus occidentalis* Nesbitt. They studied the ultrastructural and molecular characteristics of this microsporidian and also discussed its taxonomic classification. All stages of this new microsporidium are haplokaryotic and develop in direct contact with the host-cell cytoplasm. Sporogony is disporoblastic and spores are formed in eggs, immature stages, and adults of *M. occidentalis*. There are two morphological classes of spores, one with a short polar filament (3-5 coils) that measured 2:53×1:68 µm and one with a longer polar filament (8-9 coils) that measured 3:14×1:77 µm. Analysis of the small subunit ribosomal DNA indicated that this species from *M. occidentalis* is most closely related to the *Nosema/Vairimorpha* clade of microsporidia.

Choi *et al.* (2002) reported the presence of *Nosema* sp. in Cabbage White Butterfly (*Pieris rapae*) in Korea. They purified and characterized the microsporidia according to its gene structure, spore morphology and pathogenicity. They have distinguised the presence of endospores, exospores, nuclei, about 12 polar filament coils of the polar tube and posterior vacuoles by SEM and TEM study. The nucleotide sequence was determined for a portion of genomic DNA which spans the V4 variable region of the small subunit rRNA gene. Comparison with the GenBank

database for 15 other microsporidia species suggests that this isolate is most closely related to *Nosema* species. Peroral inoculation at a dosage of 10^8 spores/ml resulted in the death of all larvae prior to adult, but at lower spore dosages of 10^4-10^5 spores/ml, many adults successfully emerged. The median lethal dose (LD50) was determined to be 4.6×10^6 spores/ml and the isolate also transmitted transovarially to the progeny eggs at a frequency of 92%.

Sokolova and Lange (2002) have conducted a research work on ultrastructural study of a microsporidia *Nosema locustae* from three species of Acrididae (Orthoptera) *viz.* the migratory locust, *Locusta migratoria migratorioides* (Oedipodinae), the South American locust, *Schistocerca cancellata* (Cyrtacanth acridinae), and the grasshopper *Dichropluss chulzi* (Melanoplinae). They have described the ultrastructure of meronts, sporonts, sporoblasts and spores. They found that tubule-like structures appeared in the host cell cytoplasm during parasite sporogony. Spores were found to be of diplokaryotic type that measured 4.95±0.07 µm × 2.65±0.04 µm (Length × Width) (n=24) on fresh smears and 3.49±0.18×1.73±0.04 µm (n=10) on ultrathin sections, and had electron-dense cytoplasm in which all internal structures typical of microsporidian spores were recognizable. The polaroplast was lamellar, the endospore was 200-300 nm thick, and the exospore was 40-50 nm. The polar filament was isofilar, arranged in 17-18 coils.

Bhat and Nataraju (2004) reported a new Microsporidium from Lamerin breed of the silkworm *B. mori* L. They carried out the detailed ultrastructural and immunological study to compare the new strain with standard strain of *N. bombycis*.

Sokolova *et al.* (2004) studied the morphology, ultrastructural characteristics and development of a microsporidium *Thelohania solenopsae* isolated from the red imported fire ant *Solenopsis invicta*. For the first time, they introduced ablative laser microbeam microscope, in the easy detection to microsporidia. They also isolated individual octospores and megaspores into groups of 8–20 from fixed smears of the infected ants and subjected to subsequent PCR analysis. Octopore measured 2.6±0.49 µm × 1.5±0.19 µm (Length × Width) in ultrathin section with polar filament arranged in one row of 9–12 coils. Megaspore was larger measured 5.5±1.02 µm × 2.8±0.73 µm (Length × Width) with an anisofilar polar filament arranged in 19-23coils and two

to four rows. The SSU rDNA nucleotide sequences revealed that octospores and megaspores were identical.

Chakrabarti and Manna (2006) compared the morphological differences in the *Nosema* sp. spores isolated from four different economically important silkworm *viz. Bombyx mori* L. (Mulberry), *Anthereae mylitta* Drury (Tasar), *Philosamia ricin*i Boisd (Eri) and *Antheraea assamensis* Westwood (Muga) in India by using light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Kriukov *et al.* (2006) isolated a microsporidium from the adipose tissue of dragonfly *Aeshna viridis* larvae and studied the ultrastructure. The size of fresh spore was measured 6.9±0.09 µm × 4.1±0.08 µm (Length × Width). The nucleus of the spore was elongated with an anisofilar polar filament composed of 10-11 coils both in anterior and posterior sides. From Siberia, this was the first description of microsporidian infection in insects from the order Odonata.

Sokolova *et al.* (2006) from South Siberia reported the presence of octospore microsporidium *Systenostrema alba* Larsson 1988 in the nymphs of Dragonfly *Aeshna viridis* (Odonata, Aeshnidae). Microsporidian infection was restricted to the fat body and adipose tissues. The infected tissues were fragile, easily disintegratable and contained numerous shiny white nodules of 10-50 µm diameters. Average length and width of the fresh uninucleate spores were measured as 6.1±0.07 µm and 3.0±0.04 µm (n=50) respectively. However, the average length and width of the spores on the Giemsa stained smears were measured as 4.6±0.06 µm and 2.8±0.05 µm (n=50) respectively. The polar filament was anisofilar with 10-11 anterior coils (thicker filament) and 10-11 posterior (thinner filament) coils. Sporophorous vesicles measured 12.3±0.23 µm × 11.9±0.20 µm. Phylogeny was developed on the basis of small subunit rDNA sequence analysis placed *Systenostrema alba*, as the sister taxon to a clade consisting of *Thelohania solenopsae*, *Tubulinosema ratisbonensis*, and *Tubulinosema acridophagus.*

Rao *et al.* (2007) isolated seven new strain of microsporidian parasites isolated from the mulberry silkworm, *Bombyx mori* L. and differentiated them on the basis of RAPD-PCR. They also studied the pathologenicity, morphological and ultrastructural

characterization of the spores. Pathogenicity and virulence of the microsporidia were different from each other and directly dependent on dose. The shapes of the spores were ranged from oval to elongate, and 8-16 coils were found in polar filament arranged in one or two rows.

Chen *et al.* (2009b) have carried out a study on morphological, molecular, and phylogenetic characterization of *N. ceranae*, a microsporidian parasite isolated from the European honey bee, *A. mellifera.* The ultrastructural features indicate that *N. ceranae* possesses all of the characteristics of the genus *Nosema*. Spores of *N. ceranae* measured approximately 4.4×2.2 µm on fresh smears. The number of coils of the polar filament inside spores was 18-21. Polymerase chain reaction (PCR) signals specific for *N. ceranae* detected the infection not only in primary infection site, the midgut, but also in the tissues of hypo-pharyngeal glands, salivary glands, Malpighian tubules, and fat body. Maximum parsimony analysis of the small subunit rRNA gene sequences showed that *N. ceranae* appeared to be more closely related to the wasp parasite, *N. vespula,* than to *N. apis*, a parasite infecting the same host.

Tokarev *et al.* (2010) described a new microsporidium, *Crispospora chironomi* isolated from the gut epithelium of *Chironomus plumosus* and studied their life cycle, ultrastructure, and molecular phylogeny. Two types of sporogonies were appeared in the host larvae *viz.* polysporoblastic sporogonies, where spherical monokaryotic spore were formed within a thick-walled capsule and in disporoblastic sporogonies, diplokaryotic oval spores (2.5×1.5 µm) were formed. Phylogenetic analysis (SSU rDNA) showed the position of the new microsporidium corresponding to Class Terresporidia

Zhu *et al.* (2011) conducted a research work in which they isolated microsporidia from *Phyllobrotica armata* Baly in China and studied their detailed structure and molecular features. Light microscopy revealed that spores were long-oval and measured 4.7×2.6 µm (Length × Width) on fresh smears. Electron microscopy of mature spores revealed that exospore was thick (approximately 40 nm), polar tube with 13-14 polar filament coils which was the characteristic of genus *Nosema*.

Laarif *et al.* (2011) isolated a microsporidian species of *Nosema* strain from the potato tuber moth, *Phthorimaea operculella*. They characterized the microsporidium according to its ultrastructure and morphological characteristics by transmission electron microscopy. The ultrastructural study revealed the presence of 12 polar filament coils in the mature spore and the isolate was identified by molecular means.

Liu *et al.* (2012) studied the morphology, chromosomal karyotype and molecular characterization of a new microsporidium, *Vairimorpha* sp. isolated from the silkworm *Bombyx mori* in Shandong, China. The fresh spores were long oval and measured 3.4×1.6 µm (Length × Width) on fresh smears. Ultrastructure of the spore showed 13-15 polarfilament coils in the polar tube, a diplokaryon and a posterior vacuole which is the characteristics of *Vairimorpha* sp. Chromosome bands were separated by pulsed field gel electrophoresis. The complete rRNA gene of the isolate got sequenced which was 4231 bp long. Phylogenetic analysis was done based on SSU rRNA gene and LSU rRNA gene which revealed that this novel microsporidian had close relationship to the genus *Vairimorpha*.

Bhat *et al.* (2012) studied the characteristics features of microsopridian spores isolated from silkworm, *Bombyx mori* L. through electron microscopy. They named the isolated microsporidian spores as M_1 and M_2 which were measured 1.73 µm and 2.05 µm in length and 1.01 and 1.47 µm in width respectively. The number of coils in polar filament was also recorded as 10 and 12 in M_1 and M_2 respectively. They concluded that, the M1 and M2 microsporidian spores are different from each other and also from that of *N. bombycis*. The standard strain of *Nosema bombycis* spores were oval in shape with approximately 1.87 µm in length and 1.39 µm in width.

Chen *et al.* (2012) reported a mirosporidium *Nosema* sp. MPr from cabbage butterfly *Pieris rapae* in Zhenjiang City, Jiangsu Province, China. The mature spores were oval in shape measured 3.8±0.3 µm × 2.0±0.2 µm (Length × Width) in size. Phylogenetic tree was constructed on the basis of SSU rRNA and LSU rRNA gene sequences which confirmed that the isolated microsporidium was a member of *Nosema* genus, and so named as *Nosema* sp. MPr.

Ovcharenko *et al.* (2013) isolated a new microsporidium, *Orthosomella lipae* from adults of weevil, *Liophloeus lentus* Germar, 1824 (Coleoptera: Curculionidae) in southern Poland. The spores were present in the outer ovariole sheath, trophic chambers, oocytes, somatic tissues and eggs of the weevil. Live spores were rod-shaped and measured 4.2±0.4 (3.4-5.2) µm in length and 1.6±0.2 (1.2-2.0) µm in width. The sizes of the spores were reduced in Giemsa stained smear which were measured 3.1±0.2 (2.5-4.7) µm in length and 1.6±0.2 (1.2-1.9) µm in width. Meronts were monokaryotic and the nucleus measured 1.7-1.8 µm in diameter. Cytoplasm of meronts contained ribosomes and rough cytoplasmic reticulum. Sporonts were binucleate which underwent binary fission and produced sporoblasts. Mature spores were uninucleate with electron-lucid endospore (150-180 nm thick) and two-layered exospore (40-50 nm thick). The isofilar polar filament was 60-70 nm in diameter; coiled in 12-14 turns and arranged in one row. Posterior vacuole was about 200 nm in diameter and was filled with electron dense granules.

Andreadis *et al.* (2013) reported a new genus and species of Microsporidian parasite, *Takaokaspora nipponicus* from the rock pool mosquito, *Ochlerotatus japonicas japonicus* and studied their morphology, development, transmission cycle and molecular characterization. Diplokaryotic stage was found in horizontally-infected hosts that measured 4.3 µm × 2.0 µm (Length × Width) whereas haploid stage was found in vertically-infected larvae with unpaired nuclei throughout, producing rosette-shaped sporogonial plasmodia. Infection was appeared in the fat body and selected segments of the abdomen which appeared as white mass. They sequenced small subunit ribosomal DNA (SSU rDNA) of two morphologically similar microsporidia isolated from *Ochlerotatus japonicas japonicus* and *Ochlerotatus hatorii.*

Steele and Bjornson (2014) isolated a new microsporidian species *Nosema adaliae* from the two-spotted lady beetle *Adaliabi punctata* L. in Nova Scotia, Canada and its relationship was established to other microsporidia that infect coccinellids. They described the tissue pathology, ultrastructure and molecular characteristics of the microsporidia. Size of the mature spores were measured as 4.25±0.09 µm × 1.82±0.03 µm (Length × Width) with an isolfilar polar filament of 10-18 coils arranged in a single row. Heavy infestation of the spores was found in flight muscles

and fat body of the lady beetle. Molecular characterization of the microsporidium showed 97% similarity to *Nosema bombi* and 96% similar to *Nosema thomsoni*, *Nosema vespula* and *Nosema oulemae*.

Yaman *et al.* (2014) isolated a new microsporidian species, *Nosema pieriae* from the cabbage butterfly, *Pieris brassicae* and studied their morphology and ultrastructural characteristics by using light microscopy and scanning electron microscopy. They also studied the small subunit rDNA analysis of the spore. The infection of pathogen was found in the gut of *P. brassicae*. Spores were having an isofilar polar filament with six coils. Meront spores were spherical in shape and measured as 3.68±0.73 µm × 3.32±1.09 µm (Length × Width) or ovoid in shape which were measured as 4.04±0.74 µm × 2.63±0.49 µm (Length × Width) whereas those of sporonts were spherical to elongate in shape and measured as 4.52±0.48 µm in length and 2.16±0.27 µm in width.

Luo *et al.* (2014) described the ultrastructual morphology, tissue pathology and molecular features of *Vairimorpha necatrix* isolated from the silkworm *Bombyx mori* L. Vertical transmission was not recorded in silkworm eggs which were confirmed by PCR analysis. They recorded two types of spores *viz.* diplokaroytic spores with 13-17 coils of polar tubes and monokaryotic spores with less coils of polar tubes. Further, phylogenetic analysis was developed by sequencing the small subunit rRNA genes of *Vairimorpha* species which confirmed that this isolate has a closer relationship to *Vairimorpha necatrix*.

2.3 INFECTIVITY OF MICROSPORIDIA ISOLATED FROM ONE INSECT PEST TO OTHER INSECTS

Kishore *et al.* (1994) conducted a study where they have collected the wild lepidopterous adult insects in and around mulberry fields and tested them for the presence of microsporidian parasites. They also studied the cross infectivity of these isolated microsporidians to silkworm, *Bombyx mori* L. and found that the microsporidians were infective to silkworm, *B. mori* L.

Solter *et al.* (2002) have conducted a study in Bulgaria, on the impact of mixed infection of three species of microsporidia *viz. Endoreticulatus, Nosema, and*

Vairimorpha, in gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae). All possible combinations of two species were administered either simultaneously or sequentially to larvae and mortality, duration of development, and larval weight at 20 days post-infection (simultaneous inoculation) or 23 days post-infection (sequential inoculation) were chosen as the outcome variables. Larvae were also dissected and the presence of each species of microsporidia and the tissues infected were recorded for each treatment. Effects of infection were dependent on both host sex and the type of exposure. Infected larvae were more likely to die than uninfected larvae, but there were no differences in mortality between single and mixed infections. When *Nosema* was administered simultaneously with *Endoreticulatus* or *Vairimorpha*, infected larvae weighed more than larvae that had single infections with either pathogen. They conclude that histological evidences in combination with the data on larval weight supports the hypothesis that competition occurred in mixed infections.

Tsai *et al.* (2003) studied the morphology of the microsporidian spores isolated from five lepidopteran insect pests *viz. Spodoptera exigua, Spodoptera litura, Plutella xylostella, Helicoverpa armigera* and *Pieris* sp. The cross infectivity of the isolated microsporidia to *S. litura* were studied. They studied the cross infectivity of the isolated microsporidia to the insect pest *S. litura*.

Rao *et al.* (2004) studied the pathogenicity, mode of transmission, tissue specificity of infection and molecular phylogeny of the three new microsporidians isolated from the silkworm, *Bombyx mori* L.

Bashir and Sharma (2008) studied the morphology, infectivity and transmission of five different microsporidia in the silkworm, *Bombyx mori* L., isolated from insect pests of mulberry and some other agricultural crops.

Singh *et al.* (2008) performed a research on microsporidia isolated from mulberry insect pests and checked their cross infectivity to silkworm, *B. mori* L. They have reported two new microsporidia from Bihar hairy caterpillar, *Spilosoma oblique* and both were found to be infective to silkworm *B. mori* L.

Chakrabarty *et al.* (2012) carried out a research work on the infectivity of various types of *Nosema* sp. on Vanya and Mulberry silkworms. They observed that,

multiplications of *Nosema* sp. were directly proportional to the larval mortality and this *Nosema* sp. was able to produce pebrine disease in their secondary host under high dose and favourable environmental conditions.

Nath *et al.* (2012) isolated five new microsporidian parasites from the silkworm *Bombyx mori* which were designated by them as NIWB-11bp, NIWB-12n, NIWB-13md and NIWB-14b. They described the spore morphology, life cycle, pathogenicity and phylogenetic relationships. The pathogenicity rate was directly depended on the dose provided and differed for each of the microsporidian variety. Among the five microsporidian species, NIWB-15mb was found to be more virulent. They studied the Phylogenetic analysis categorized NIWB-11bp, NIWB-12n and NIWB- 14b in one group while NIWB- 15mb and NIWB-13md in another group along with other *Nosema* species.

Velide and Bhagavanulu (2012) studied the effect of *Nosema* infection on the cocoon characters in the ecoraces of tasar silkworm, *Anthereae mylitta* Drury. They also studied the pathogenecity rate of the microsporidia on Andhra local and Daba and compared them with that of the respective infected ones. They found a significant impact of *Nosema* infection on larval weight, mortality of larva and pupa, number of moths emerged, percentage of infected moths, hatching % etc. Various phenotypic characters of cocoon were recorded *viz*. cocoon weight, shell length, shell weight, shell width, shell thickness, peduncle thickness, peduncle weight, peduncle length, peduncle diameter, SR % filament length, denier, reelanility and weight of the silk reeled in both infected ecoraces in the infected ecoraces.

Ghani *et al.* (2013) investigated the biological characteristics of a microsporidium isolated from Diamondback Moth, *Plutella xylostella* (PX) and checked its pathogenicity against the noctuid moths *Spodoptera litura* and *Spodoptera exigua*. All these insects were collected from cruciferous vegetable farms. A significant difference was observed in both the shape and size of the microsporidia isolated from *Plutella xylostella* (ovocylindrical) and *Spodoptera litura* (oval).

Das *et al.* (2014) conducted a study on the cross infectivity of pebrine disease from muga to eri silkworm. They have isolated the microsporidium, *Nosema antheraea* from the muga silkworm and inoculated to the second instar healthy eri

silkwom larvae in ambient temperature and relative humidity. Highest larval and pupal mortality was recorded during the period September-October (13.7%) and July-August (13.2%) respectively. In adult eri silkworm, highest *N. antheraea* infection of was recorded in May-June (63.72%). Therefore, the study confirmed the cross infectivity of pebrine disease from muga to eri silkworm.

Sharma *et al.* (2014) carried out a study on the impact of a microsporidian infection on larval and cocoon parameters of the silkworm *B. mori* L. The microsporidian spores were further studied for their morphology, pathogenicity and the transmission. The microsporidian has resulted in low larval and pupal mortality but remarkably high infection percentage in moth stage at 1×10^5 and 1×10^6 spore/ml inoculation doses. Studies also indicated that infection by the new microsporidian in mother moth did not impact the larval health and cocoon parameters in the next generation. In case of *N. bombycis*, cocoon parameters were significantly affected as the inoculum dose increased from 1×10^3 to 1×10^6 spores/ml. The larvae hatched from the eggs laid by *N. bombycis* infected moths (20 and above spores/field) did not survive up to cocooning.

2.4 MICROSPORIDIA AS BIOCONTROL AGENTS OF INSECTS

Microsporidia have potential for development as microbial insecticides, but do not produce a rapid effect and therefore slowly kill their hosts. Many species have complex life cycles involving more than one host. For the above reason, they are considered as chronic pathogen; fit more closely the paradigm for parasitoids and predators than that of microbial insecticides (Solter and Maddox, 1998). Further, the establishment of microsporidian biocontrol agents could be an integral part of the sustained control of a pest population within the appropriate environment and natural enemy complex. The significant destruction of crops and rangeland forage by grasshoppers (Hewitt and Onsager, 1983; Lockwood *et al.*, 2002) has made them the focus of intense control programs utilizing various insecticide formulations (Latchininsky and VanDyke, 2006). Further, host specificity is one of the most important factors to consider when evaluating pathogens and other organisms for use as classical biological control agents. The endosymbiont microsporidian parasites play an important role as a biocontrol agent in the natural regulation of insect populations as they reduced fecundity and shorter life span (Nataraju *et al.*, 2005; Solter and

Becnel, 2000). For the above purpose, spore based biopesticides have been developed, registered and successfully used in long-term biocontrol agent against insect pests (Raina *et al.*, 1987). Several species of microsporidia have been isolated from various species of grasshopper and locust of which *Paranosema locustae* is the only microsporidium registered as a biocontrol agent for grasshopper control (Johnson, 1997; Lockwood *et al.*, 1999; Solter *et al.*, 2012). They developed in the USA as a long-term microbial control agent of grasshoppers (Henry and Oma, 1981). The pathogen was first introduced from North America and later became established in grasshopper communities in areas of north-western Patagonia and the western Pampas of central Argentina (Bardi *et al.*, 2012; Lange and Azzaro, 2008).

The grasshopper outbreaks reported to have ceased in areas where *P. locustae* established successfully in contrast to similar areas without the microsporidium (Lange and Cigliano, 2010). From an extensive study in China it has observed that the microsporidium *P. locustae* persist at a sites of 15,000 ha for a minimum of 9-10 years. Again, the infections of *P. locustae* in large grasshopper populations result in a sustained presence of the disease which is important in reducing future outbreaks (Miao *et al.*, 2012). From a study it was reported also that the administration of *P. locustae* in a grasshopper population reduces the population to 30% along with 20-40% infection prevalence among the survivors (Johnson, 1997). However, when compared with the chemical insecticides which typically achieve 70-95% control, *P. locustae* efficacy may be perceived to be inadequate by users who desire consistent and rapid grasshopper suppression (Vaughn *et al.*, 1991). But, *P. locustae* could be useful in providing long-term grasshopper suppression in environmentally sensitive areas where rapid and significant pest population reductions at a minimal cost are not the primary concern. Apart from this, Mewis *et al.* (2003) reported microsporidian infection in the insect pest species *Hellula undalis* for the first time. They investigated the biology; pathogenicity and transmission of this pathogen to estimate its potential use as a microbiological control agent.

Chapter 3

Prevalence of Microsporidian Infection in the Insect Collected from Mulberry and other Agricultural Crops

PREVALENCE OF MICROSPORIDIAN INFECTION IN THE INSECT PESTS COLLECTED FROM MULBERRY FIELD AND OTHER AGRICULTURAL CROPS

3.0 INTRODUCTION

The insects comprise approximately half of the total living species and are found in almost all the ecosystems because of their diverged life forms. They are the most successful group on this planet due to their wide array of adaptive abilities. Insects are associated intimately with the human survival in that certain insects damage human health and that of their domestic animals and others adversely affect the agriculture and horticulture. However, many of the insects play a critical role in maintaining the ecosystem. Therefore, the insects are divided into two major groups' *viz.* beneficial and harmful insects. Insects are beneficial as they pollinate crops, act as natural enemies of damaging pests, and produce useful products for human beings. They are also harmful as major pests of food crops and vectors for transmission of deadly diseases, as well as cause damage to our urban infrastructure, environment, forest and natural resources and sometimes interfere with international trade, commerce and economic affairs (Ikawa *et al.*, 2002)

Insects are essential to the following ecosystem functions:

- ✓ Nutrient recycling, via leaf-litter and wood degradation, dispersal of fungi, disposal of carrion and dung, and soil turnover.
- ✓ Plant propagation, including pollination and seed dispersal.
- ✓ Maintenance of plant community composition and structure, via phytophagy, including seed feeding.
- ✓ Food for insectivorous vertebrates, such as many birds, mammals, reptiles, and fish.
- ✓ Maintenance of animal community structure, through transmission of diseases of large animals, and predation and parasitism of smaller ones.

3.0.1 Microbial Diseases in Insects

The Insecta is one of the largest taxonomic classes of living organisms which is probably accommodating more than one million species of insects. They are

vulnerable to a number of microbial diseases caused by viruses, bacteria, fungi and protozoans. Studies on control of diseases in insect production systems historically focused mostly on the two main insect species maintained in large quantities: the honeybee (*A. mellifera*) and the silkworm (*B. mori*), which have a long history of domestication. There are decades and even centuries of experience with diseases in these two insect species (Bassi, 1835, 1836; Pasteur, 1870; James and Li, 2012) and therefore these animals are models to understand insect diseases. Many insect pathogens (e.g. almost all viruses) are very host specific, while others (e.g. some bacteria and fungi) have a broader host range.

Some insect pathogens can create epidemics with high mortality and striking symptoms on the hosts (Roy *et al.*, 2006) while others may cause chronic or asymptomatic infections for an extended period of time with scarce or no direct mortality (Lange and Lord, 2012). Among all the microbial infection in insects, microsporidian infection is important because this entomopathogen is diversely prevalent in insect species and yet many are to be reported. The microsporidian parasite shares a common origin with the fungi and causes a dreaded disease in insects called microsporidiosis. Therefore, it is essential to understand the biology of the pathogens that infect the insects.

3.0.2 Incidence of microsporidia in insects

Microsporidia are found to infect members of almost all the animal phyla, but especially common in arthropods. Again, they are considered as the natural pathogens of insects under field conditions (Larsoson, 1988). Currently, 93 of the 200 described genera of microsporidia have an insect as the type host (Becnel and Andreadis, 2014). Most of these genera are distinguished based on the description of one sporulation sequence and one spore type with limited or no information on transmission or molecular sequence data. There are approximately 5-30 million insect species estimated in the world (Hevel, 2005) and the microsporidian parasites causes disease in both beneficial and harmful insects (Sprague and Becnel, 1999). Furthermore, the parasites have the potential to acts as natural bio-control agent to regulate insect populations in agriculture, horticulture and forest sectors (Onstad and Carruthers, 1990; Tanada and Kaya, 1993). Again, they are considered to be useful as long-term

regulators of pests and contribute toward the prevention and/or suppression of pest outbreaks (Bjørnson and Oi, 2014).

The microsporidia produce chronic infections in host populations by causing suppressed pupal weight and significant reductions in size, fecundity and shorter life span of host (Bauer and Nordin, 1988; Anderson and Giacon, 1992). However, the chronic and debilitating nature associated with the infections makes microsporidia important pathogens of beneficial arthropods that are used for biological pest control (Bjørnson and Oi, 2014). The microsporidia are transmitted either by vertical or horizontal, or by both means, depending on species-specific microsporidium–host interactions (Becnel and Andreadis, 1999; Goertz *et al.*, 2007). Due to this, microsporidia can rapidly establish and spread within host populations. However, the infection of the parasite is relatively unnoticed in insect population, until a high prevalence reduces productivity and performance (Kyei-Poku *et al.*, 2011).

The microsporidian parasite is highly prevalent in insect populations; however there is lack of in depth study regarding the prevalence rate of this parasite in specific insect host. In this chapter, the prevalence of microsporidian parasites was determined in the insects collected from mulberry and other agricultural crops. The study provides a clear idea about the occurrence of the pathogen in various insect populations.

3.1 MATERIALS AND METHODS

3.1.1 Study areas

Plantation in Mulberry and horticulture garden of BBAU campus and nearby agricultural fields of Lucknow was selected for the study. A total of eight varieties of mulberry (*Morus indica*) plants *viz.* S-1635, AR-12, S1, TR 10, AR 14, BR 2, S 13, S 146 and many agricultural crops were investigated for the collection of insect pests.

3.1.2 General Survey and collection of insect pests

A survey was carried out for two years from January, 2013 to June, 2016 to investigate the incidence of different types of insect pest infesting the mulberry plants and other vegetation. The insects were collected by standard insect collection techniques (Fenemore and Prakash, 1992) and brought to the laboratory for screening and isolation of microsporidia.

3.1.3 Methods of Collection/sampling methods

The larvae were collected mainly by handpicking method with the help of forceps whereas adult butterflies and honey bees were collected with the help of an insect collection net (with a light and strong handle of 1 m length, rim of 0.3 m diameter made of heavy wire and a nylon net bag with open mesh) (Fig 3). After collection, the insects were transferred immediately to plastic boxes with perforated lids and bought to the laboratory for further identification and examination for microsporidian infection.

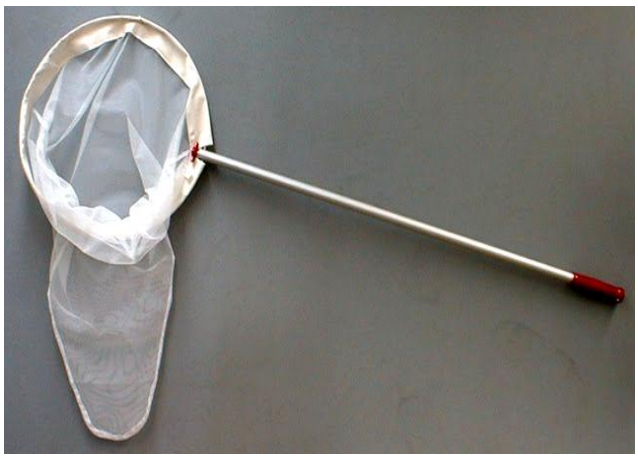


Figure 3: Images of Aerial insect collecting net

3.1.4 Identification of collected insect pests

The collected insects were identified with the help of literature surveys and online insect identification guide (Gasse, 2013; BugGuide).

3.1.5 Screening of Insect Pests for Microsporidial infection

3.1.5.1 Homogenization

The abdomen of each insect was separated and weight was measured. The abdomen was then homogenized individually in 0.6% K_2CO_3 (4ml/g) solution and investigated for the presence of microsporidian spores.

3.1.5.2 Microscopic examination

The smear was prepared from the respective homogenate and observed for the presence of microsporidian spores under light microscope at 400X. If the samples

were found negative for the microsporidian infection, the samples were discarded whereas in case of positive infection the samples were processed for purification of spores.

3.1.5.3 Isolation and purification

If spores were present in the homogenized tissues, they were isolated and purified by following standard procedures. For purification of the microsporidium, the method given by Chakrabarty *et al.* (2013) was followed with some modifications. The homogenate was allowed to settle for 2 min and filtered through double layered muslin cloth. The filtrate was transferred to 2ml centrifuge tube for further purification. For the above purpose, the filtrate was centrifuged at 5000 rpm for 15 min. and after centrifugation the pellet was suspended in sterilized distilled water and thoroughly mixed on cyclomixer. The obtained suspension was then centrifuged at 8000 rpm for 15 min and the process was repeated 3 times by adding sterilized distilled water to the sediment. The final sediment obtained was suspended in minimal volume of sterilized distilled water and stored at 4º C.

3.1.6 Prevalence of microsporidian infection in Insects

The prevalence percentage of microsporidian infection in insects was calculated by the following formula:

$$\text{Infection rate of microsporidia (\%)} = \frac{\text{No. of infected insects}}{\text{Total no. of collected insects}} \times 100$$

3.1.6.1 Intensity of microsporidian infection in insects

The intensity of microsporidian infection was measured by counting the spores per infected insect (spore load) using a Neubaur haemocytometer (German Fine Optik) as described by Cantwell (1970). The photographs of a haemocytometer depicted in Figure 3.1. Based on the concentration of microsporidian spores per individual, the infection level was categorized as chronic (1×10^5 - 1×10^8 spores/ml) which is designated as +++, medium (1×10^3 - 1×10^5 spores/ml) which is designated as ++, and acute ($<1\times10^3$ spores/ml) level of infection which is designated as +.

$$\text{Number of spores/ml} = \frac{\text{Total spores counted} \times 4 \times 1000000}{80}$$

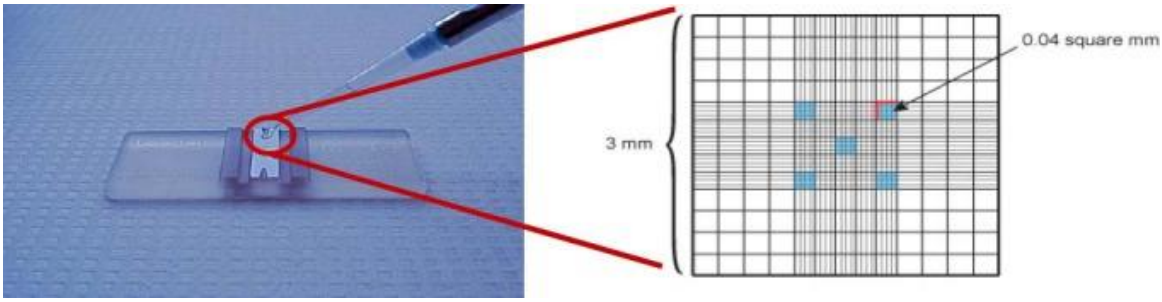


Figure 3.1: Images of Haemocytometer used for the quantification of microsporidian spores

3.2 RESULTS

Studies on microsporidia and their occurrences in insect pests of mulberry and agricultural crops, is a new area of research in Indian scenario. In this study, an attempt was made to investigate the occurrence of microsporidia in the insect pests in the Lucknow.

A total of 34 different varieties of insects were collected from the vegetation of mulberry and nearby agricultural crops of horticulture garden of Babasaheb Bhimrao Ambedkar University, Lucknow. These insects belonged to 5 orders *viz.* Lepidoptera, Hymenoptera, Orthoptera, Coleoptera and Hemiptera. The lepidopteran insects were most frequently available in the collection sites followed by Orthopteran, Hymenopteran, Coleopteran and Hemipteran insects data. Among 34 different insect species collected during the study, 5 insect species were selected for further investigations in the present study on the basis of the availability of these insect species in the study sites and the intensity of microsporidian infection in these insects.

3.2.1 Insect Pests Selected for the Study

In the present investigation, five insect pests were selected for the study based on their abundant availability and the intensity of harbored microsporidian infection in these insects. The host plants of the selected insect pests are given in Table 3. A brief description of the insects selected for the present study is as follows:

***Danaus chrysippus* (Linnaeus, 1758):** It is one of the common Nymphalid butterflies of India popularly known as Plain Tiger butterfly or African Monarch. They occur in

scrub jungles, dry deciduous forests, humid sub-tropical forests preferring areas of moderate to heavy rainfall. The most common food plants of the common tiger are milkweed species *viz. Calotropis gigantean*, small herbs, twiners and creepers from the family Periplocaceae and Asclepiadaceae. These butterflies are widely distributed in Asia and Africa. In India, *D. chrysippus* is one of the most important pests appears to be vital importance as pollinators by virtue of their wider distributions. They are endangered locally due to accidental destroyment of host plants, use of pesticides in agriculture, housing development etc.

Scientific classification

Kingdom- Animalia
Phylum-Arthropoda
Class-Insecta
Order-Lepidoptera
Family-Nymphalidae
Genus-*Danaus*
Species- *D. chrysippus*

***Catopsilia florella* (Fabricius, 1775):** It is a butterfly of the Pieridae family. It is found in Africa (including Madagascar), the Canary Islands, Sri Lanka, India, Burma, and China. The host plants of the butterfly include legumes from the *Cassia* genus *viz. Cassia javanica,* and *Cassia fistula,* also feeds on plants from *Sesbania* and *Senna* genus *viz.* on *Senna occidentalis, Senna septentrionalis, Senna petersiana.*

Scientific classification

Scientific classification
Kingdom: Animalia
Phylum: Arthropoda
Class: Insecta
Order: Lepidoptera
Family: Pieridae
Genus: *Catopsilia*
Species: *C. florella*

***Melanitis phedima* (Cramer, 1780):** The butterfly is commonly known as Dark Evening Brown butterfly that found flying at dusk. The flight of this species is erratic. They are found in South Asia. They feed on *Ischaemum semisagittatum* and a variety of grasses of the genera *Andropogon, Cymbopogon, Pennisetum, Setaria, Oplismenus compositus, and Bambusa arundinacea* (Kunte, 2006)

Scientific classification

Kingdom: Animalia
Phylum: Arthropoda
Class: Insecta
Order: Lepidoptera
Family: Nymphalidae
Genus: *Melanitis*
Species: *M. phedima*

***Apis mellifera* (Linnaeus, 1758):** It is commonly known as western honey bee or European honey bee which is native to Europe, western Asia, and Africa. Human introduction of *A. mellifera* to other continents started in the 17th century, and now they are found all around the world, including East Asia, Australia and North and South America. They prefer habitats that have an abundant supply of suitable flowering plants, such as meadows and gardens.

Scientific classification

Kingdom: Animalia
Phylum: Arthropoda
Class: Insecta
Order: Hymenoptera
Family: Apidae
Genus: *Apis*
Species: *A. mellifera*

***Samia Cynthia ricini* (Boisduval, 1854)**

It feeds upon the leaves of castor bean, and is known for the production of eri silk, and is often referred to by the common name eri silk moth. Tree of Heaven *Ailanthus* is quite their favourite food. They also thrive on cherry, laburnham, lilac, rose, plum, apple etc.

Scientific classification

Kingdom: Animalia
Phylum: Arthropoda
Class: Insecta
Order: Lepidoptera
Family: Saturniidae
Genus: *Samia*
Species: *S. Cynthia*

Table 3: Selected insect pests and their host plants

Insect pest	Designation of Microsporidia isolated from respective insect	Host plants
Danaus chrysippus	M-Dch	Common milkweed like madar plant, Ficus, Sweet Potato
Catopsilia florella	M-Cfl	Feeds on plant belongs to leguminosae family *Viz.* Red gram, pea and bean plants
Melanitis phedima	M-Mph	Variety of grasses of the genera *Andropogon, Setaria*
Apis mellifera	M-Ame	Feed on a variety of flowering plants Sunflower, Marigold, bee balm flower etc.
Samia cynthia ricini	M-Scy	Castor leaves, cherry leaves

3.2.2 Prevalence of Microsporidian Infection in Insects

The prevalence of microsporidian infection and their intensity in 34 different insect species are depicted in Table 3.1.

3.2.1 Order Lepidoptera

A total of 13 different species of butterfly and moths were collected from the insect collection sites. The insects belonging to order Lepidoptera were again categorized into 6 families *viz.* Nymphalidae, Pieridae, Papilionidae, Saturniidae, Sphingidae and Erebidae.

Family: Nymphalidae

The collected butterflies belonging to Nymphalidae family were *D. chrysippus* (Plain tiger butterfly), *D. genutia* (Striped tiger butterfly), *M. leda leda* (Common Evening brown butterfly), *M. phedima* (Dark evening brown butterfly) and *J. almana* (Peacock pancy butterfly). Among these, all the insects were found positive with the microsporidian infection except *J. almanac*. The plain tiger butterflies were most commonly available. In the present study a total of 185 plain tiger butterflies were collected, out of which 62 were found to be infected with the microsporidia; therefore the prevalence percentage of the microsporidian infection in the respective butterfly was found to be 33.51 %. A total of 27 *D. genutia*, 20 *M. leda leda* and 79 *M. phedima* butterflies were collected, out of which 3, 1 and 20 butterflies specimens in the respective group were found to be positive for the microsporidian infection. Therefore, the prevalence of microsporidian infection in *D. genutia* (Striped tiger butterfly), *M. leda leda* and *M. phedima* was calculated as 11.11%, 6 % and 25.31 % respectively (Table 3.1).

Family: Pieridae

Four different species of butterflies' *viz. C. florella, C. Pomona, C. pyranthe* and *E. brigitta* were collected that belonged to the family Pieridae. Among these, microsporidian infection was not detected in the butterfly *C. Pomona* and *E. brigitta* where as positive microsporidian infection was observed in the butterfly *C. florella*

and *C. pyranthe*. A total of 86 *C. florella* butterflies and 36 *Catopsilia pyranthe* butterflies were collected, out of which 21 *C. florella* and 5 *C. pyranthe* butterflies were found to be infected with the microsporidian infection. Hence, the prevalence percentage of microsporidian infection in *C. florella* and 36 *C. pyranthe* butterflies were calculated as 24.42% and 13.88% respectively.

Family: Papilionidae

A total of 23 lime swallowtail butterflies (*P. demoleus*) were collected and all were found to be negative for the microsporidian infection.

Family: Saturniidae

A total of 52 Indian eri silkworms (*S. cynthia ricini*) were collected out of which 17 were found infected with microsporidia. So, the prevalence of microsporidian infection in Eri silkworms was calculated as 32.69%.

Family: Sphingidae

A total of 16 lesser death's head hawk moth (*A. styx*) were collected and all were found negative with the microsporidian infection.

Family: Erebidae

A total of 68 common grass moths (*Caenurgina erechtea*) were collected and all were found negative with the microsporidian infection.

3.2.2 Order Hymenoptera

A total of 6 different species of hymenopteran insects were collected from the insect collection sites. The collected hymenopteran insects belonged to 4 families *viz.* Apidae, Vespidae, Scoliidae and Formicidae.

Family: Apidae

The collected hymenopteran insects that belonged to Apidae family were *Apis mellifera* (Western honeybee) and *Xylocopa violacea* (European carpenter bee). A total of 107 *Apis mellifera* were collected, out of which 56 were found to be positive

with the microsporidian infection. A total of 20 *Xylocopa violacea* were collected, out of which only 3 were found positive with microsporidian infection. Therefore, the prevalence percentage of microsporidian infection in Western honey bee was calculated as 52.33% whereas the value was recorded as 15.00% in case of the European carpenter bee.

Family: Vespidae

A total of 96 *Polistes flavus* (Yellow jacket wasp) were collected, out of which 18 specimens were found positive with the microsporidian infection. The prevalence percentage of microsporidian infection in *P. flavus* was measured as 18.75%.

Family: Scoliidae

A total of 28 *Scolia soror* (Hairy flower wasp) were collected, out of which 4 specimens were found positive with the microsporidian infection. The prevalence percentage of microsporidian infection in *S. soror* was measured as 14.28%.

Family: Formicidae

The collected hymenopteran insects that belonged to Formicidae family were *Lasius niger* (Black garden ant) and *Dorylus helvolus* (Driver ant). A total of 60 *Lasius niger* were collected and all the insects were found to be free from the microsporidian infection. Further, a total of 35 *Dorylus helvolus* insects were collected, out of which 3 specimens were found positive with the microsporidian infection. Therefore, the prevalence percentage of microsporidian infection in *Scolia soror* was measured as 8.57%.

3.2.3 Order Orthoptera

A total of 8 different species of orthopteran insects were collected from the insect collection sites. These collected insects belonged to 4 different families *viz.* Acrididae, Pyrgomorphidae Tettigoniidae and Gryllidae.

Family: Acrididae

The collected orthopteran insects that belonged to Acrididae family were *Acrida ungarica* (Cone-headed grasshopper), *Melanoplus sanguinipes* (Migratory

grasshopper) and *Melanoplus packardii* (Packard grasshopper). Microsporidian infection was detected in all these grasshoppers; however the intensity of the infection was quite low. A total of 70 *Acrida ungarica*, 26 *Melanoplus sanguinipes* and 28 *Melanoplus packardii* were collected, out of which 11, 1 and 2 specimens in the respective groups were found positive with the microsporidian infection. Therefore, the prevalence percentage of microsporidian infection in *Acrida ungarica*, *Melanoplus sanguinipes* and *Melanoplus packardii* was calculated as 15.71 %, 3.84 % and 7.14 % respectively.

Family: Pyrgomorphidae

A total of 23 *Poekilocerus pictus* (Painted Grasshopper) and 41 *Atractomorpha crenulata* (Tobacco grasshopper) were investigated for the presence of microsporidian infection but all the insects were found negative for the infection.

Family: Tettigoniidae

A total of 18 *Caedicia simplex* (Common garden katydid) insects were collected and investigated for the presence of microsporidian infection. All were found negative for the infection.

Family: Gryllidae

A total of 22 *Acheta domesticus* (House cricket) *Neocurtilla hexadactyla* (Northern mole cricket) were investigated for the presence of microsporidian infection but all the insects were found negative for the infection.

3.2.4 Order Coleoptera

A total of 5 species of coleopteran insects were collected from the insect collection sites. These collected insects belonged to 5 different families *viz.* Scarabaeidae, Coccinellidae, Meloidae, Dytiscidae and Curculionidae.

Family: Scarabaeidae

A total of 65 *Phyllophaga latifrons* (June beetle) insects were collected and investigated for the presence of microsporidian infection and all were found negative for the infection.

Family: Coccinellidae

A total of 81 *Coccinella septempunctata* (7 spotted Lady beetle) were collected, out of which 13 specimens were found positive with the microsporidian infection but the intensity of infection was very low. The prevalence percentage of microsporidian infection in *Scolia soror* was measured as 16.04 %.

Family: Meloidae

A total of 55 *Hycleus phaleratus* (Blister beetle) insects were collected and investigated for the presence of microsporidian infection and all were found negative for the infection.

Family: Dytiscidae

A total of 11 *Cybister fimbriolatus* (Predaceous diving beetle) insects were collected and investigated for the presence of microsporidian infection and all were found negative for the infection.

Family: Curculionidae

A total of 108 *Phyllobius* sp. (Common leaf weevil) insects were collected and investigated for the presence of microsporidian infection and all were found negative for the infection.

3.2.5 Order Hemiptera

Family: Cicadidae

A total of 16 *Neotibicen auletes* (Dusk singing cicada) insects were collected and investigated for the presence of microsporidian infection and all were found negative for the infection.

Family: Aphididae

A total of 110 *Brevicoryne brassicae* (Cabbage aphid) insects were collected and investigated for the presence of microsporidian infection but all were found to be negative for the infection.

Figure 3.2: Photographs of the collected insects during the study

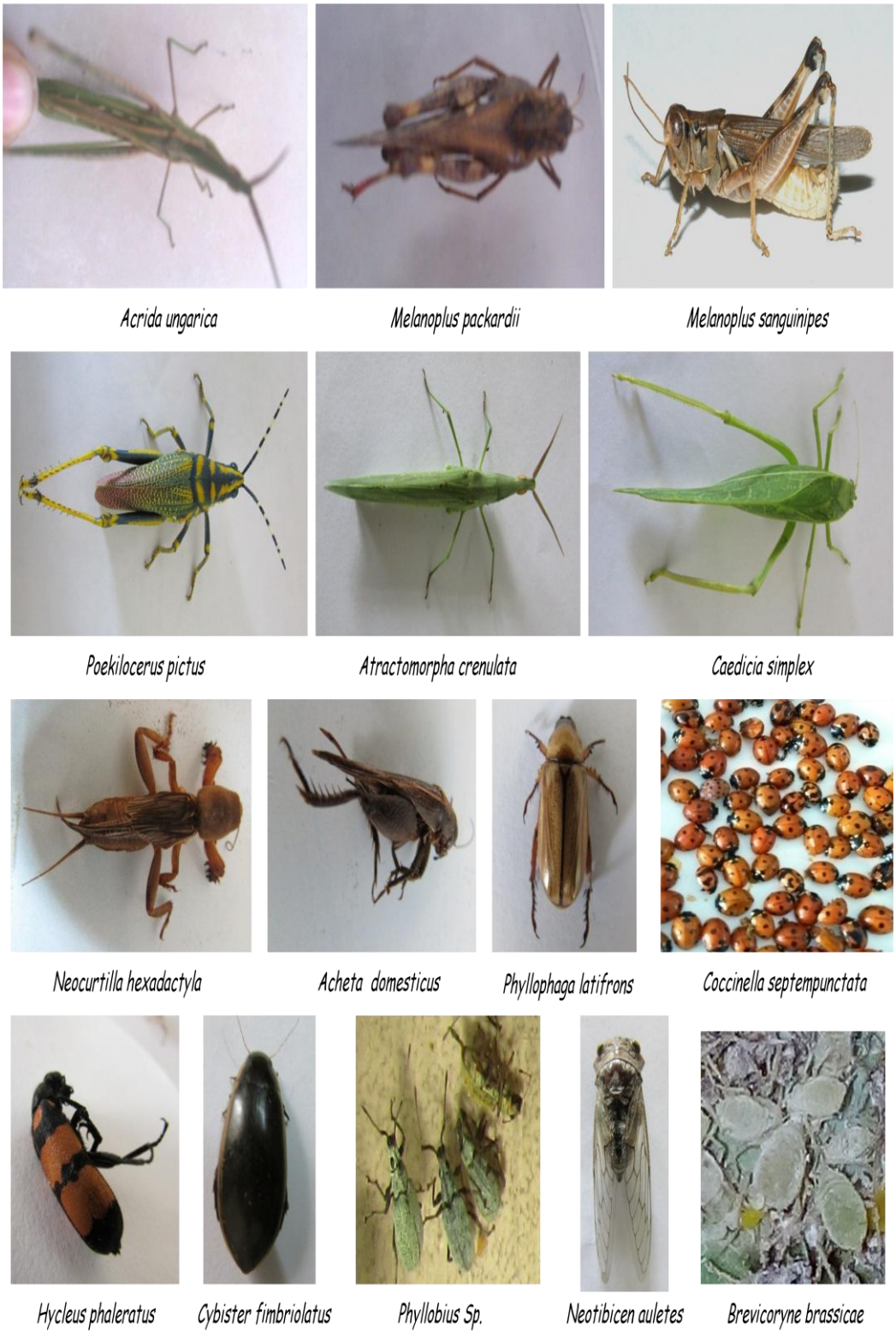


Figure 3.2: Photographs of the collected insects during the study

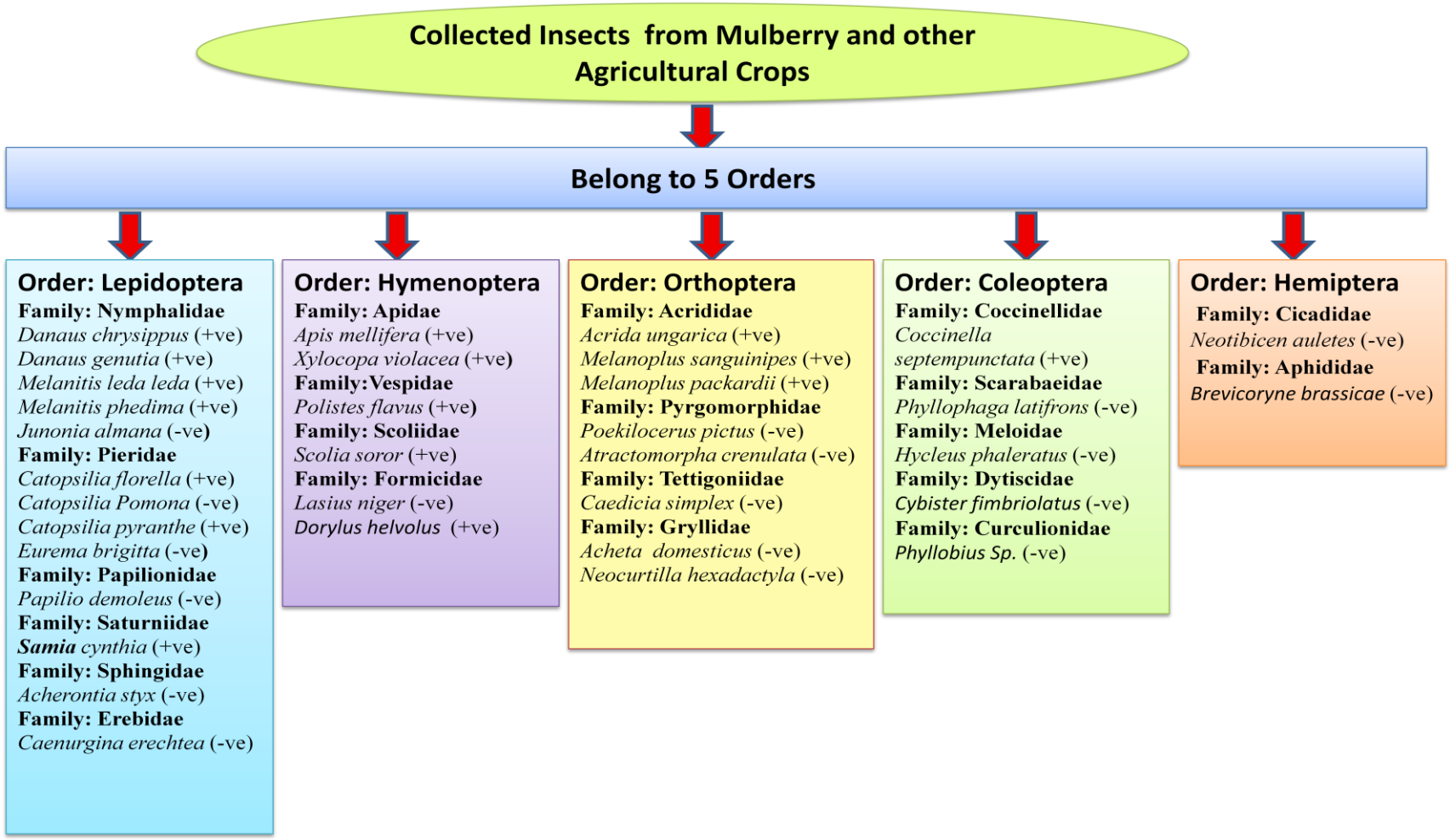


Figure 3.3: Categorization of collected insects to their respective taxonomic order and family (+ve: Positive microsporidian infection, -ve: Negative microsporidian infection)

Table 3.1: Prevalence of microsporidian infection in insect species

Sr No	Insect species	Common Name	Order	Family	No. of Specimen collected	No. of infected specimen	Prevalence %	Intensity
1	*Danaus chrysippus* (Linnaeus, 1758)	Plain tiger butterfly	Lepidoptera	Nymphalidae	185	62	33.51	++
2	*Danaus genutia* (Cramer 1779)	Striped tiger butterfly	Lepidoptera	Nymphalidae	27	3	11.11	++
3	*Melanitis leda leda* (Linnaeus, 1758)	Common Evening Brown butterfly	Lepidoptera	Nymphalidae	20	1	6.00	+
4	*Melanitis phedima* (Cramer, 1780)	Dark Evening Brown butterfly	Lepidoptera	Nymphalidae	79	20	25.31	+
5	*Junonia almana* (Linnaeus, 1758)	Peacock pancy butterfly	Lepidoptera	Nymphalidae	26	0	0	-
6	*Catopsilia florella* (Fabricius, 1775)	African emigrant butterfly	Lepidoptera	Pieridae	86	21	24.42	+++
7	*Catopsilia pomona* (Fabricius, 1775)	Lemon emigrant butterfly	Lepidoptera	Pieridae	20	0	0	-
8	*Catopsilia pyranthe* (Linnaeus, 1758)	Mottled emigrant butterfly	Lepidoptera	Pieridae	36	5	13.88	+++

Sr No	Insect species	Common Name	Order	Family	No. of Specimen collected	No. of infected specimen	Prevalence %	Intensity
9	*Eurema brigitta* (Cramer, 1780)	Small grass yellow butterfly	Lepidoptera	Pieridae	18	0	0	-
10	*Papilio demoleus* (Linnaeus, 1758)	Lime swallowtail butterfly	Lepidoptera	Papilionidae	23	0	0	-
11	*Samia cynthia ricini* (Drury, 1773)	Indian eri silkworm	Lepidoptera	Saturniidae	52	17	32.69	+++
12	*Acherontia styx* (Westwood, 1847)	Lesser death's head hawk moth	Lepidoptera	Sphingidae	16	0	0	-
13	*Caenurgina erechtea* (Cramer, 1780)	Common Grass Moth	Lepidoptera	Erebidae	68	0	0	-
14	*Apis mellifera* (Linnaeus, 1758)	Western honeybee	Hymenoptera	Apidae	107	56	52.33	+++
15	*Xylocopa violacea* (Linnaeus,1758)	European Carpenter Bee	Hymenoptera	Apidae	20	3	15	++
16	*Polistes flavus* (Cresson, 1868)	Yellow jacket wasp	Hymenoptera	Vespidae	96	18	18.75	+
17	*Scolia soror* (Smith, 1855)	hairy flower wasp	Hymenoptera	Scoliidae	28	4	14.28	+++

Sr No	Insect species	Common Name	Order	Family	No. of Specimen collected	No. of infected specimen	Prevalence %	Intensity
18	*Lasius niger* (Linnaeus, 1758)	Black Garden Ant	Hymenoptera	Formicidae	60	0	0	-
19	*Dorylus helvolus* (Linnaeus, 1764)	Driver ant.	Hymenoptera	Formicidae	35	3	8.57	+
20	*Acrida ungarica* (Herbst, 1786)	Cone-headed Grasshopper	Orthoptera	Acrididae	70	11	15.71	+
21	*Melanoplus sanguinipes* (Gurney, 1962)	Migratory Grasshopper	Orthoptera	Acrididae	26	1	3.84	+
22	*Melanoplus packardii* (Scudder, 1878)	Packard grasshopper	Orthoptera	Acrididae	28	2	7.14	+
23	*Poekilocerus pictus* (Fabricius, 1775)	Painted Grasshopper	Orthoptera	Pyrgomorphidae	23	0	0	-
24	*Atractomorpha crenulata* (Fabricius, 1793)	Tobacco grasshopper	Orthoptera	Pyrgomorphidae	41	0	0	-
25	*Caedicia simplex (Walker, 1869)*	*Common garden katydid*	Orthoptera	Tettigoniidae	18	0	0	-
26	*Acheta domesticus* (Linnaeus, 1758)	House cricket	Orthoptera	Gryllidae	22	0	0	-
27	*Neocurtilla hexadactyla* (Perty, 1832)	Northern mole cricket	Orthoptera	Gryllidae	17	0	0	-

Sr No	Insect species	Common Name	Order	Family	No. of Specimen collected	No. of infected specimen	Prevalence %	Intensity
28	*Phyllophaga latifrons* (LeConte, 1856)	June Beetle	Coleoptera	Scarabaeidae	65	0	0	-
29	*Coccinella septempunctata* (Linnaeus, 1758)	7 spotted Lady beetle	Coleoptera	Coccinellidae	81	13	16.04	+
30	*Hycleus phaleratus* (Pallas, 1781)	Blister Beetle	Coleoptera	*Meloidae*	55	0	0	-
31	*Cybister fimbriolatus* (Aubé,1838)	Predaceous Diving Beetle	Coleoptera	Dytiscidae	11	0	0	-
32	*Phyllobius Sp.*	Common Leaf Weevil	Coleoptera	Curculionidae	108	0	0	-
33	*Neotibicen auletes* (Germar, 1834)	Dusk Singing Cicada	Hemiptera	Cicadidae	16	0	0	-
34	*Brevicoryne brassicae* (Linnaeus, 1758)	Cabbage aphid	Hemiptera	Aphididae	110	0	0	-

3.3 DISCUSSION

Among the entomopathogens, microsporidia are highly ecologically diverse parasites that have gained prominence both as pathogens and potential biocontrol agents. They also represent a model system for research on host-parasite interactions which is the least studied group as far as their utilization for biological control of noxious insect pests is concerned. Further, research on microsporidian interactions with insect host is well studied only in case of *N. bombycis* which causes pebrine disease in mulberry silkworm, *B. mori*. Therefore, microsporidians directly affect the economy of any country by devastating the economy of sericulture industry. However, apart from these studies, microsporidian prevalence among insect pests of mulberry and agricultural crops is least explored area of research in Indian scenario. This thesis embodies the experimental results of a pioneering study on the association of microsporidia in insect pests of mulberry and agricultural crops. In this study, an attempt was made to investigate the occurrence of microsporidia in different species of insect pests of mulberry and agricultural crops inhabiting the agro ecosystems of Lucknow region of India.

3.3.1 Occurrence of microsporidia among insect pests of mulberry and agricultural crops

Microsporidian infection was generally found to be prevalent in insect population of Lucknow region in India. However, the infections often go unnoticed and their role in population dynamics of insects is often not recognized, because microsporidia do not cause dramatic epizootics such as those caused by fungi and viruses. There is less literature available about the prevalence of microsporidian parasites in insects. Therefore, the present study surely gives a brief idea about the distribution and prevalence percentage of microsporidian infection in the insect population commonly inhabiting the mulberry and agricultural fields.

In the present investigation, 34 species of insects belonging to five orders (Lepidoptera, Hymenoptera, Orthoptera, Coleoptera and Hemiptera) were screened for infection by microsporidian parasites. The infection was reported in the four orders except Order Hemiptera. Among these 34 species of insects, microsporidian

infection was recorded in 16 insect species. The occurrence of microsporidia in some insect species *viz. M. phedima, C. florella, X. violacea, P. flavus, D. helvolus, A. ungarica* and *C. septempunctata* are reported for the first time. As far as study on the microsporidia is concerned, this is the first study conducted on the prevalence of microsporidia in Uttar Pradesh State of India. The results of the present study showed that all butterfly species inhabiting the surveyed areas had microsporidian infection except the Peacock pancy butterfly, *J. almana;* whereas infection was not found in the two moth species investigated i.e. Lesser death's head hawk moth, *A. styx* and Common Grass moth, *C. erechtea*. Studies by several workers have also indicated that microsporidian infection is very common in Lepidopteran insects (Kishore *et al.*, 1994; Choi *et al.*, 2002; Singh *et al.*, 2008; Bashir *et al.*, 2011; Bhubaneswari and Surendra Nath 2015a, 2015b). In the butterflies and moth species investigated in the present study the maximum prevalence percentage of microsporidian infection was recorded in *D. chrysippus* (33.51%) followed by *S. cynthia ricini* (32.69%), *M. phedima* (25.31%), *C. florella* (24.42%), *C. pyranthe* (13.88%), *D. genutia* (11.11%), and *M. leda leda* (6%). The result of the present study is more or less supported by the previous study by Bashir *et al.* (2011) where they have screened the lepidopteran insect pests of mulberry and agricultural crops collected from in and around Mysore, Karnataka, India and reported the prevalence percentage of microsporidian infection as 25.60%, 25.33%, 20.00%, 19.31% and 18.40% respectively in insect species *Spilosoma obliqua*, *Catopsilia pyranthe*, *Pieris rapae*, *Diaphania pulverulentalis*, and *Catopsilia crocale*. These authors have also reported that some of the lepidopteran insects were negative for microsporidian infections *viz. Sesamia inferens*, *Phytomyza atricornis*, *Eupterote mollifera*, *Terias hecabe*, *Laphygma exigua* and *Colias eurytheme*. Eveleigh *et al.* (2012) also reported that, the annual spruce budworm (*Choristoneura fumiferana*) mortality associated with microsporidium *N. fumiferanae* was ≤15% of all mortality in reared specimens and was positively correlated with but generally less than 30% of annual *N. fumiferanae* prevalence.

Earlier studies have reported the occurrence of microsporidian infection in the butterflies *C. pomona*, *P. demoleus* and *Eurema* sp. (Smitha, 2011), however in the present investigation, the microsporidian infection was not detected in these butterflies. It may be due to the fact that microsporidian infections show area

specificity. Solter *et al.* (1997) reported five biotypes of microsporidia in European populations of *Lymantria dispar* but none were found in North American population of *L. dispar*. In the present study, microsporidian infection was not found in the peacock pancy butterfly, *J. almanac*. A similar report was also given by Smitha (2011) wherein she reported that the peacock pancy butterfly, *J. almanac* was free of microsporidian infection.

Though microsporidian parasites commonly occur in the hymenopteran insects, the prevalence of microsporidian parasites is well studied only in the European honey bee *A. mellifera*. In the present study, among all the insects studied, the highest prevalence percentage of microsporidian infection was recorded in *A. mellifera* (52.33%). This result is supported by a previous study of Lotfi *et al.* (2009) who reported that the highest infection rate of *Nosema* in *A. mellifera* was observed in May (83.3%) whereas 59.5% of *Nosema* infection was recorded during spring season and a minimum of 3.3% of infection was observed in summer season. However, the microsporidian infection in honey bee was generally higher in the rainy season than in the dry season, with the highest prevalence of 71.4 % during the months of August (Dawet *et al.*, 2016).

Their study clearly showed, that the prevalence of microsporidian infection in insects varried according to seasons. In a study by Aydin *et al.* (2006) it was reported that, 23.8% of honey bee colonies were infected with *Nosema apis*. Further, the study concluded that nosemosis disease in *A. mellifera* reduced worker longevity by 22-44% which in turn reduced the honey production, therefore causing heavy economic losses (Kang *et al.*, 1976; Fries *et al.*, 2003; Higes *et al.*, 2010, 2010a). In case of severe infection of nosemosis in honey bee, it lead to Colony Collapse Disorder (Chen and Huang, 2010; Gisder *et al.*, 2010).

Among the orthopteran and coleopteran insects collected during the present investigation, some of the insects were found positive for the microsporidian infection but the intensity of the infection was very low. Therefore, these insects were not selected for further investigations.

3.4 CONCLUSION

The Microsporidia is a diverse phylum of eukaryotic endoparasite infecting host groups from all major taxa in all environments ranging from those infecting pest and beneficial insects, to important parasites of humans. This chapter describes the prevalence percentage of microsporidian infection in the insect species collected from mulberry and other agricultural crops. Out of the 34 different insect species collected and screened, the microsporidian infections were found in 16 different species of insects. Out of these infected insects, only five insect species were selected for the study on the basis of the availability of these insect species in the study sites and the intensity of microsporidian infection in those insects. The selected insect species include four lepidopteran insects *viz. D. chrysippus, C. florella, M. phedima* and *S. cynthia ricini* and one hymenopteran insects *A. mellifera.* Amog these insects, highest prevalence percentage of microsporidian infection was recorded in *A. mellifera* (52.33%) followed by *D. chrysippus* (33.51%) followed by *S. cynthia ricini* (32.69%), *M. phedima* (25.31%) and *C. florella* (24.42). The microsporidian infection is consistent in lepidopteran insects as compared to others, however highest prevalence of infection is found in *A. mellifera*. This is the first report of microsporidian infection in the insect pest inhabiting the Lucknow region of Uttar Pradesh. From the present study, we can conclude that microsporidian parasites are prevalent in insects and yet many more need to be explored further.

Chapter 4

Microscopic Study of Microsporidian Spores Isolated from Insect Pests of Mulberry and other Agricultural Crops

MICROSCOPIC STUDY OF MICROSPORIDIAN SPORES ISOLATED FROM INSECT PESTS OF MULBERRY AND OTHER AGRICULTURAL CROPS

4.0 INTRODUCTION

The microsporidian spores are ubiquitous and are capable of infecting many organisms including insects, fish, mammals and even other parasites. The microsporidia exhibit a number of unique features, some of them are reductions while others are structural innovations. The microsporidian parasites are important in agriculture as pathogens of beneficial insects such as silkworm and honey bees (Becnel and Andreadis, 1999; Rao *et al.*, 2004) or as biological control agents for use against medical or agricultural pests (Lockwood *et al.*, 1999; Solter and Becnel, 2000; Briano and Williams, 2002). The early descriptions of microsporidians are mainly based on spore morphology and lack ultra structural details but in the recent methods of identification, it is necessary to use at least a minimum of ultrastructural characters to identify the microsporidia (Larsson, 1988). Instead of typical mitochondria, the microsporidia possess mitosomes, which are remnants of mitochondria and possess unstacked Golgi apparatus (Williams *et al.*, 2002; Vávra, 2005).

4.0.1 Life cycle

The life cycles of entomogenous microsporidia range from relatively simple to the extremely complex. Spores are the only life cycle stage of microsporidia that survives outside the host cell. This is the infectious stage which normally reaches the host through the gut even if other routes of transmission are known to occur. Again, due to their durability and structural complexity, the spores are essential for microsporidian recognition and classification. The spores contain uninucleate or binucleate sporoplasm with a long and coiled extrusion apparatus called as polar filament (Vávra and Larsson, 1999).

4.0.2 Reproduction Modes and Life cycle stages

During spore germination, the polar filament injects the sporoplasm into a host cell which undergoes a reproduction cycle and terminates with the production of spores (Vávra and Lukeš, 2013).

In most microsporidia, two sequences of reproduction follow each other: an initial vegetative reproduction (merogony) yielding daughter cells (merozoites) with the potential either to repeat merogony or to enter the second reproductive phase, the production of spores (sporogony). However, some microsporidia, like the *Nosema* species, reproduce weakly in the sporogony, where each cell initiating sporogony yields only two spores (Brooks *et al.*, 1985). Species with a weak sporogony reproduce more efficiently during the merogony, where several cycles of divisions might follow each other. The merogonial reproduction has never been observed in a small number of microsporidia, and the only reproduction known to occur is the production of spores. This is characteristic for the genera *Chytridiopsis* (Larsson, 1993) and *Metchnikovella* (Vivier & Schrével, 1973) and a few other related genera. The budding like processes have been observed during the merogonial divison in the genus *Chytridiopsis* which probably indicates the beginning of sporogony (Tonka *et al.*, 2010).

In most of the microsporidia, both merogony and sporogony division proceed by binary fission resulting into two daughter cells from each mother cell. In some cases, the daughter cells adhere to each other, forming chains of cells. The life cycle stages of microsporidia are ultra structurally unique and distinct from other spore-forming micro-organisms and hence play a critical role in determining the taxonomic position (Larsson, 1986).

Keeping the above in view, in this chapter, the developmental cycle of five microsporidia isolated from the selected insect pests *viz. D. chrysippus, C. florella, M. phedima, A. mellifera, S. cynthia ricini* were studied. Mid-gut tissue of insects is the first site to be infected and, therefore designated as the primary site of infection among the tissues.

4.1 MATERIALS AND METHODS

4.1.1 Light microscopy

Light microscopy is an essential tool for the identification of microsporidian stages and is best examined by phase contrast microscope. The microsporidian spores isolated from insect pests of mulberry and agricultural crops were subjected to morphological characterization following standard method as described by Fujiwara (1980) and compared with the spores of the standard strain *N. bombycis.*

The size of the fresh microsporidian spores were measured accurately by using Nikon NSBR software under Nikon Type-104 phase contrast microscope at 400X without shrinkage and drastic distortion, often caused by fixation and staining. The information about shape, color, movement and refractivity of spores was obtained during the study (Hazard *et al.*, 1981). Data were pooled to calculate the mean size of the spores.

4.1.2 Ultrastructural Study

4.1.2.1 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) is a useful technique for the investigation of surface structure of biological samples. The surface patterns of the microsporidian spores like surface undulations and ridges are best resolved by scanning electron microscopy.

In the present study, the microsporidian spores isolated from insect pests of mulberry and agricultural crops were subjected to scanning electron microscopy by following the standard method (Undeen, 1997). The scanning electron microscopy of the microsporidia was done at the University Science Instrumentation Centre (USIC), Babasaheb Bhimrao Ambedkar University, Lucknow.

Protocol for processing of spores for SEM

- ✓ **Primary Fixation**: A small piece of the insect gut infected with microsporidian spores were fixed in 2.5% (v/v) glutaraldehyde ($C_5H_8O_2$) in 0.1 M phosphate buffer, pH 7.4 for 24 hours.
- ✓ **Primary Wash:** The sample was then washed thrice (each 5 minute duration) in 0.1 M phosphate buffer pH 7.4 to remove the fixative.
- ✓ **Secondary Fixation:** Post fixation of the sample was done in 1% osmium tetroxide (OsO_4), pH 7.4 for 1 hour followed by three times washing in the same buffer. The osmium tetroxide stored in a dark bottle at 4° C.
- ✓ **Secondary Wash:** The sample was then washed thrice (each 5 minute duration) in 0.1 M phosphate buffer pH 7.4 to remove the fixative.
- ✓ **Dehydration:** SEM samples were dehydrated by immersing the samples for 30 min each in fresh solutions of 30%, 50%, 70%, 90% and 100% acetone.
- ✓ **Mounting:** The dried sample was then mounted on copper stub using carbon tape.
- ✓ **Sputter Coating:** Finally, a thin layer of metals (gold and palladium) was applied over the sample using an automated sputter coater.
- ✓ **Photography:** Mounted spores were scanned under a JEOL JSM-6490LV scanning electron microscope, and the sizes of the spores were measured. Photographs were taken at different magnifications.

4.1.2.2 Transmission Electron Microscopy

The ultra-thin section of the spore provides the information about the length, arrangement and number of coils of polar filaments which is important for the identification of microsporidia at the generic level as it varies among different microsporidia. In the present study, the isolated spores of microsporidia from individual insects were processed for Transmission Electron Microscopy at Sophisticated Analytical Instrument Laboratory (SAIF), All India Institutes Medical

Sciences, New Delhi, India. Transmission Electron Microscopy of *N. bombycis* spores was carried out for comparison of the ultrastructure of *Nosema* spores with that of the microsporidian spores isolated from insect pests of mulberry and other agricultural crops.

Protocol for processing of spores for TEM (Reynolds, 1963)

- ✓ A small piece (1×1 mm) of infected gut tissue of insect was fixed in 2.5% glutaraldehyde and 2% paraformaldehyde (PF), in 0.1 M phosphate buffer (pH 7.4) for 24 hr at 4°C.
- ✓ The sample was then washed thrice (each 5 minute duration) in 0.1 M phosphate buffer, pH 7.4 to remove the fixative.
- ✓ Samples were submitted at Sophisticated Analytical Instrument Laboratory, AIIMS, New Delhi for further processing.
- ✓ Ultrathin sections of the samples were cut on a Reichert-Jung Ultracut E ultramicrotome and stained using uranyl acetate and lead citrate. The sections were then observed under a TECNAI (Fei, Electron Optics) Transmission Electron Microscope, operated at 200 KV.

4.2 RESULTS

Studies of microsporidia in insect pests of mulberry and other agricultural fields are a new area of research in Indian scenario. In the present study, 34 different insect species were collected, out of which 16 species of insect species were found positive for the microsporidian infection. Five insect pests *viz. D. chrysippus, C. florella, M. phedima, A. mellifera, S. cynthia ricini* were selected for the study and the microsporidia isolated from these insect pests were designated as M-Dch, M-Cfl, M-Mph, M-Ame, M-Scy after the first letter of the genus and two letters of specific names respectively. Similarly, the designation Nbo was used for the standard strain of microsporidia, *N. bombycis* which were isolated from the mulberry silkworm *B. mori* L. Further, the isolated microsporidia were characterized in detail using light microscopy, scanning electron microscopy and transmission electron microscopic studies.

4.2.1 Light Microscopic Study

The spores isolated from these insects were subjected to light microscopic studies and their shape, mean length and width were recorded. The live micosporidian spores were easily detected by their characteristic Brownian movement. The light microscopy revealed that all the five different microsporidia *viz.* M-Dch, M-Cfl, M-Mph, M-Ame, M-Scy were oval in shape with variable sizes. The live microsporidian spores showed translucent properties with high refractivity whereas the germinated spores were easily distinguished from the live spores and observed as black empty spores under phase contrast microscope. Besides this, the microsporidian spores exhibited clear and bright glare with a characteristic halo effect. The nucleus and dividing stages of the microsporidia were also clearly observed under the phase contrast microscope.

The sizes of the fresh microsporidia were larger than the fixed microsporidia. The size (Length × Width) of the fresh microsporidia, M-Dch (Figure 4), M-Cfl (Figure 4.2), M-Mph (Figure 4.4), M-Ame (Figure 4.6) and M-Scy (Figure 4.8) were measured as 4.06±0.12 µm × 2.29±0.08 µm, 4.32±0.11 µm × 2.33±0.06 µm, 5.35±0.09 µm × 3.26±0.11 µm, 4.35±0.10 µm × 2.80±0.07 µm and 3.52±0.12 µm × 2.22±0.10 µm respectively; whereas the size of Nbo spores were measured as 3.27±0.10 µm × 2.09±0.07 µm (Table 4, Figure 4.10). Again, it was observed that, the mean sizes of the above five microsporidia were smaller than that of the spore size of *N. bombycis*. The germinated microsporidian spores were left with an empty shell and were visible as blackish in colour whereas the non germinated spores showed high refractivity (Figure 4.2). It was further observed that the refractivity of the microsporidian spores get reduced to some extent when fixed in Nigrosin stain solution. The images of M-Dch, M-Cfl, M-Mph, M-Scy, M-Ame and *N. bombycis* spores fixed in Nigrosin solution are decpicted in Figure 4.1, 4.3, 4.5, 4.7, 4.9 and Figure 4.11 respectively. The microsporidian spores are often found in pairs, suggesting disporoblastic sporogony.

Table 4: Morphometric details of fresh microsporidia

Insect pests	**Isolated Microsporidia**	**Shape**	**Size (µm)**	
			Length±SD	**Width±SD**
D. chrysippus	M-Dch	Oval	4.06±0.12	2.29±0.08
C. florella	M-Cfl	Oval	4.32±0.11	2.33±0.06
M. phedima	M-Mph	Oval	5.35±0.09	3.26±0.11
A.mellifera	M-Ame	Oval	4.35±0.10	2.80±0.07
S. cynthia ricini	M-Scy	Oval	3.52±0.12	2.22±0.10
B. mori	*Nosema bombycis* (Nbo)	Oval	3.27 ± 0.10	2.09 ± 0.07

Table 4.1: Morphometric details of fixed microsporidia by SEM

Insect pests	**Isolated Microsporidia**	**Size (µm)**		**Texture of spore surface**
		Length±SD	**Width±SD**	
D. chrysippus	M-Dch	3.83±0.09	2.16±0.10	Smooth
C. florella	M-Cfl	4.09±0.11	2.19±0.17	Smooth
M. phedima	M-Mph	5.22±0.18	3.12±0.07	Smooth
A.mellifera	M-Ame	4.01±0.17	2.67±0.08	With Ridges and Furrows
S. cynthia ricini	M-Scy	3.45±0.16	2.13±0.15	Smooth
B. mori	*Nosema bombycis* (Nbo)	3.04±0.08	1.97±0.07	Smooth

4.2.2 Ultrastructural Study

4.2.2.1 Scanning Electron Microscopy (SEM)

The surfaces of the microsporidian spores were clearly visible under scanning electron microscope. The sizes of the microsporidia get reduced to some extent by the

fixative and dehydrating agents used to fix the spores for SEM study. Further, the scanning electron microscopy revealed that all the five microsporidian spores *viz.* M-Dch, M-Cfl, M-Mph, M-Ame, M-Scy were different from each other in terms of their shape, size and spore surface (Table 4.1). These microsporidian spores were also different from the standard strain of *N. bombycis* strain.

M-Dch

The average size (Length × Width) of the M-Dch spores under scanning electron microscope was measured as 3.83±0.09 µm × 2.16±0.10 µm. The exospore wall of the M-Dch microsporidia was smooth. Small groupings of immature M-Dch spores were clearly observed and such type of groupings were also observed under the phase contrast microscope (Figure 4.12 A). Protrusion of the spore wall was observed in some of M-Dch mature spores which indicated that, the spore was going to germinate (Figure 4.12 B).

M-Cfl

The mean size (Length × Width) of the M-Cfl microsporidia under scanning electron microscope was measured as 4.09±0.11 µm × 2.19±0.17 µm. The exospores of the microsporidia M-Cfl were smooth with no ornamentation. The developmental stages of M-Cfl spores *viz.* meront and spore stages were clearly observed. The meronts were rounded in shape whereas the spore stages were oval to ovo-cylindrical in shape. The Gut epithelial cells of *C. florella* showed heavy intensity of microsporidian infection filled with M-Cfl spores (Figure 4.13 and Figure 4.14).

M-Mph

The average size (Length × Width) of the microsporidia M-Mph microsporidia under scanning electron microscope was measured as 5.22±0.18 µm × 3.12±0.07 µm. The exospores wall of the spore was smooth. Both meront and spore stages of M-Mph spores were observed in the intestinal epithelial cells of *M. phedima* (Figure 4.15 and Figure 4.16).

M-Ame

The mean size (Length × Width) of the microsporidia M-Ame under scanning electron microscope was measured as 4.01±0.17 µm × 2.67±0.08 µm. The honey bee gut observed under SEM was fully covered with microsporidian spores with a high intensity of spore load. The developmental stages of M-Ame spores were clearly observed under SEM. The meront stages were rounded in shape whereas the spore stages were oval in shape (Figure 4.17). Further, the M-Ame spores undergoing binary fission were also observed under SEM (Figure 4.18). The exospores of the M-Ame microsporidia were sculptured with deeper ornamentation with ridges and furrows (Figure 4.19).

M-Scy

The average sizes (Length × Width) of the microsporidia M-Scy under scanning electron microscope were measured as 3.45±0.16 µm × 2.13±0.15 µm. The exospores of the M-Scy microsporidia were smooth and distinct with no ornamentation (Figure 4.20). The M-Scy spores were oval in shape and mature spore showed spore wall protrusion (Figure 4.21).

Nbo

The mean sizes (Length × Width) of the microsporidia *N. bombycis* (Nbo) under scanning electron microscope (SEM) were measured as 3.04±0.08 µm × 1.97±0.07 µm. The exospores of the Nbo microsporidia were smooth (Figure 4.22 and Figure 4.23).

4.2.2.2 Transmission Electron Microscopy (TEM)

The developmental stages of the five microsporidian spores *viz.* M-Dch, M-Cfl, M-Mph, M-Ame, M-Scy were observed under transmission electron microscopy and compared to the standard strain of *N. bombycis*. Transmission electron microscopic observation confirmed that the developmental stages (meront, sporont, sporoblast) and mature spores of all the above five microsporidia were developed in direct contact with the host cell cytoplasm. The internal structure of these

microsporidia showed characteristics typical of microsporidia. The width of exospores of all the five microsporidia was thinner than the endospore. Further, from the studies of internal structure of the mature microsporidia, the spores were identified upto the level of genus. The microsporidian species were identified after the descriptions of Weiser (1961) and Maurand (1973), using the additional observations of Maurand (1975) and Larsson (1999).

M-Dch

The gut epithelium of *D. chrysippus* showed both merogony and sporogonic development M-Dch spores. The meronts enveloped by a single cytoplasmic membrane containing ribosomes (Figure 4.24). Electron microscopic observations of mature M-Dch microsporidia confirmed that the oval spores contain a diplokaryon measuring 481 nm in diameter. The spore wall was composed of two layers, a thin, electron-dense exospore and a thick electron-transparent endospore. The exospore was surrounded by a thin, irregular layer of granular material. The thickness of the exospore and endospore were measured as 25 nm and 160 nm respectively. The isofilar polar filament consisted of a regular coil in a single layer with 11 turns (Table 4.2, Figure 4.25). The diameter of the mature polar filament coil was measured 45 nm. Further, the spore contained a posterior vacuole and an anchoring disc whereas the polaroplast was not visible. The angle of tilt between the anterior polar filament coil and spore axis was 42°. The ultrastructural features of M-Ame spores confirmed that it belongs to the genus *Nosema*.

M-Cfl

The meront, sporon, sporoblast and early spore stages were observed in the gut epithelium of *C. florella.* Meront stage of the spore was divided by binary fission (Figure 4.26). Electron microscopic observations of M-Cfl microsporidia confirmed that the spores were ovo-cylindrical in shape. The mature spore contained two rounded nucleus which was 500 nm in diameter. Further, the M-Cfl microsporidia possessed two uniformly thickened exospores and endospore wall. The thickness of the exospore and endospore were measured as 25 nm and 122 nm respectively. The polar filament was isofilar and had 14 coils (Table 4.2, Figure 4.27). The polar

filament coils measured 31 nm in diameter. The anchoring disc was located in the apical region of the spore in an eccentric position relative to the spore axis, rendering bilateral asymmetry to the spore. Again the posterior vacuole was observed at the posterior end of the spore whereas the polaroplast was not visible clearly. The angle of tilt between the most anterior and posterior polar filament coil to the spore axis was 90°. The above ultrastructural features of M-Cfl spores confirmed that it belongs to the genus *Nosema*.

M-Mph

The meront stages of the M-Mph microsporia was rounded (2.5-3 µm in diameter) with a nucleus. The cytoplasm contains numerous free ribosomes and poorly developed endoplasmic reticulum.. In contrast to meront stage, the larger sporonts possessed a better developed rough endoplasmic reticulum. The sporoblst stage was recognized due to the presence of thickened spore wall (Figure 4.28). Electron microscopic observations of M-Mph microsporidia confirmed that the spores were ovo-cylindrical in shape. The mature spore contained one centrally placed nucleus which was 628 nm in diameter. The thickness of the exospore and endospore were measured as 27 nm and 146 nm respectively. The polar filament was isofilar and had 11 coils (Table 4.2, Figure 4.29). The polar filament coils measured 33 nm in diameter. A small posterior vacuole was observed at the posterior end of the spore. The angle of tilt on most anterior to posterior coil was 90°. The ultrastructural features of M-Mph spores revealed that it possessed all of the characteristics of a typical microsporidia and belong to a genus other than *Nosema* as the spore contained a monokaryon. The above ultrastructural features of M-Mph spores confirmed that it belonged to the genus *Larssoniella*.

M-Ame

All stages of the spore were in direct contact with the host cell cytoplasm. The meront, sporon, sporoblast and early spore stages were observed in the gut epithelium of *A. mellifera.* Some empty spore walls were also observed (Figure 4.30 and Figure 4.32). Infected cells were enlarged and the cytoplasm contained a larger number of mitochondria and free ribosomes. Several mitochondria were close to and surrounded

the plasmalemma of meronts (Figure 4.31). he In cells with sporogonial phases, the rough endoplasmic content was greater The M-Ame spores were ovo-cylindrical in shape and contained two nuclei in diplokaryotic arrangement which were closely apposed in the central region of the spore and located between the polarplast and the posterior vacuole. The diameter of the nucleus was measured as 830 nm. The thickness of the exospore and endospore were measured as 24 nm and 162 nm respectively. The polar filament was isofilar consisting of 21 coils which were arranged in two rows (Table 4.2, Figure 4.33). The polar filament coils measured 41 nm in diameter. A small posterior vacuole was observed at the posterior end of the spore. The angle of tilt on most anterior to posterior coil was 55°.

Further, the ultrastructural study of M-Ame spore showed that different developmental stages, including meronts, sporonts, sporoblasts, and mature spores are found in the midgut epithelial cells. Meronts, the earliest developmental stage, had two nuclei in diplokarytic arrangement and were bound by a plasma membrane. Sporonts were elongated and oval in shape with dense cytoplasm and no visible internal structures. Sporoblasts were generally smaller than sporonts with a more clearly defined cell wall and two nuclei. The anchoring disc was located in the anterior pole of the spore. The lamellate polaroplast occupied the anterior part of the spore adjacent to the anchoring disc but was not prominent. A vacuole was located in the posterior end of the spore. The ultrastructural features of M-Ame spore confirm that it belonged to the genus *Nosema* as the spore possessed all of the characteristic features of a microsporidium that is typical of genus *Nosema*.

M-Scy

The merogonial and sporogonial developmental stages of M-Scy spore were observed in the gut epithelium of *S. cynthia ricini* (Figure 4.34). The M-Scy spores were ovo-cylindrical in shape and contained two nuclei in diplokaryotic arrangement in the central region of the spore between the polarplast and the posterior vacuole. The diameter of the nucleus was measured as 491 nm. The thickness of the exospore and endospore were measured as 22 nm and 120 nm respectively. The polar filament was isofilar consist of 12 coils which were arranged in a single rows (Table 4.2, Figure 4.35). The polar filament coils measured approximately 36 nm in diameter. A

small posterior vacuole was observed at the posterior end of the spore. The angle of tilt on most anterior to posterior coil was 62°. All these ultrastructural features of M-Scy spores matched the principle characteristics of the genus *Nosema*. Hence, the M-Scy spore belonged to the genus *Nosema*.

Nbo

The merogonial and sporogonial developmental stages of M-Scy spore were observed in the gut epithelium of *S. cynthia ricini*. Meronts were spherical to ovoid and bounded by a plamalemma which forms elongated membranous protrusion. Sporoblast were characterized by thickening of spore wall (Figure 4.36). The Nbo spores were ovo-cylindrical in shape and contained two nuclei in diplokaryotic arrangement in the central region of the spore between the polarplast and the posterior vacuole. The diameter of the nucleus was measured as 468 nm. The thickness of the exospore and endospore were measured as 27 nm and 122 nm respectively. The polar filament was isofilar consist of 14 coils which were arranged in a single rows (Table 4.2, Figure 4.37). The polar filament coils measured approximately 55 nm in diameter. A small posterior vacuole was observed at the posterior end of the spore. The angle of tilt on most anterior to posterior coil was 62°. The ultrastructural features of Nbo spores revealed the typical internal structure of *N. bombycis* spore.

Table 4.2: Ultrastructural details of microsporidia viewed under TEM

Microsporidian isolates	**No. of coils of polar filament**	**Width of Polar Tubule (nm)**	**No. of nuclei**	**Diameter of Exospore (nm)**	**Diameter of Endospore (nm)**
M-Dch	11	45	2	25	160
M-Cfl	14	31	2	25	122
M-Mph	11	43	1	27	146
M-Ame	21	41	2	24	162
M-Scy	12	36	2	22	120
N. bombycis/ Nbo	14	55	2	27	123

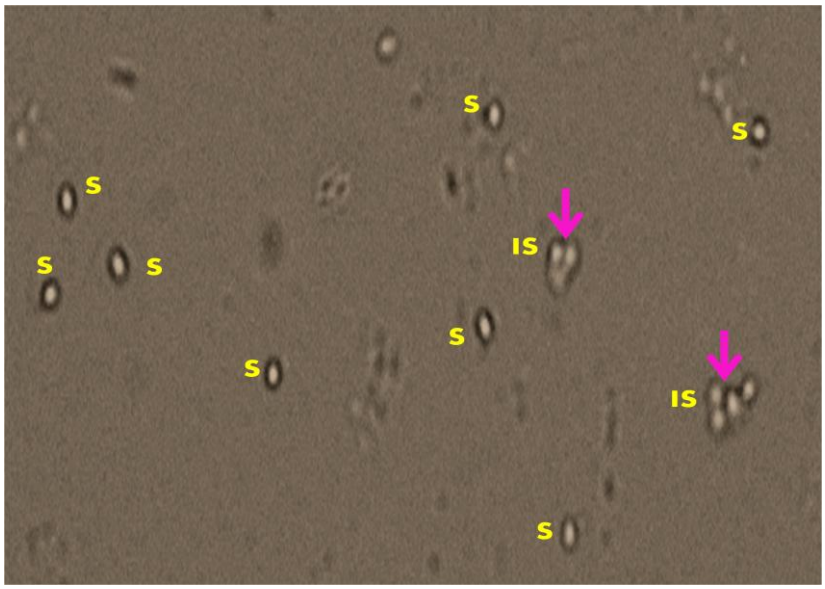


Figure 4: Light micrograph of M-Dch spores in water suspension (400X), Arrow indicates grouping of immature M-Dch spores during merogony, S: Mature Spore, IS: Immature spore

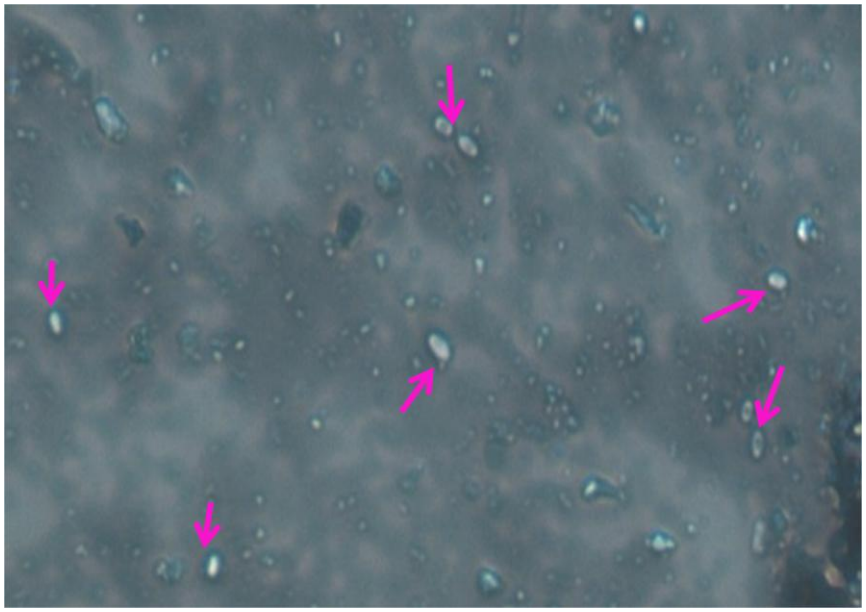


Figure 4.1: Light micrograph of M-Dch spores stained with Nigrosin stain solution (400X)

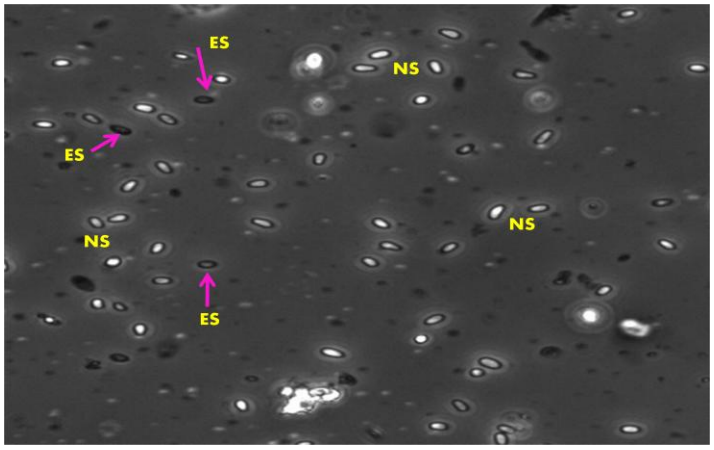


Figure 4.2: Light micrograph of M-Cfl spores in water suspension (400X), Arrow indicating the black empty spores, ES: Empty spore, NS: Non germinated spore

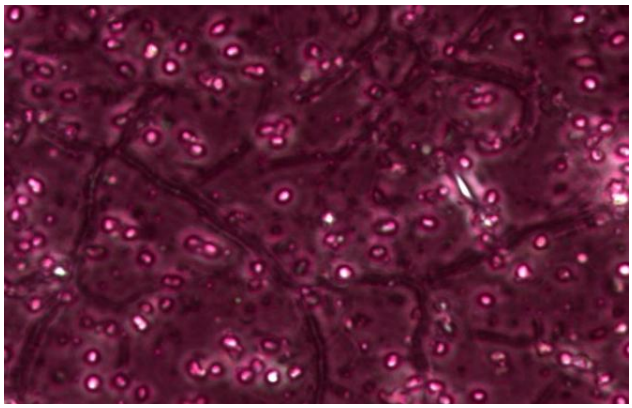


Figure 4.3: Light micrograph of M-Cfl spores stained with Nigrosin stain solution (400X)

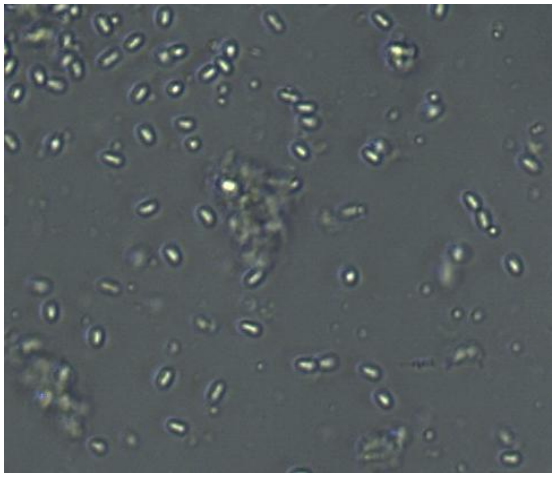


Figure 4.4: Light micrograph of M-Mph spores in water suspension (400X)

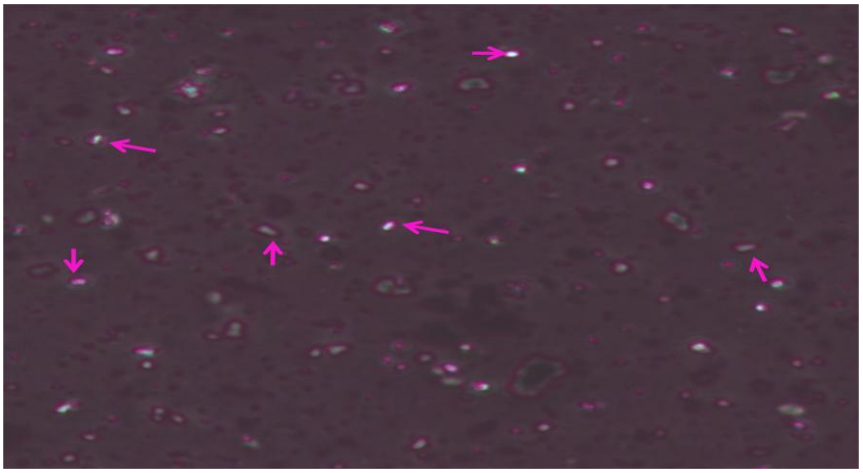


Figure 4.5: Light micrograph of M-Mph spores stained with Nigrosin stain solution (400X)

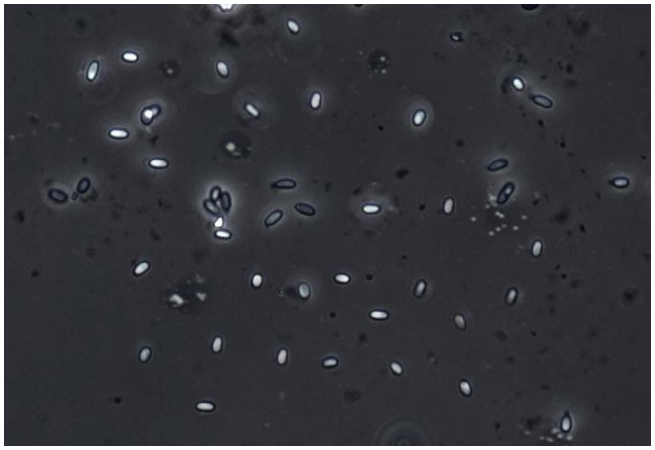


Figure 4.6: Light micrograph of M-Ame spores in water suspension (400X)

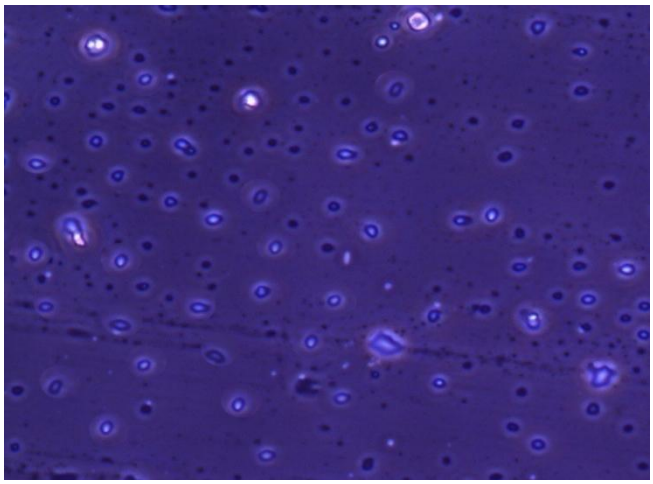


Figure 4.7: Light micrograph of M-Ame spores stained with Nigrosin stain solution (400X)

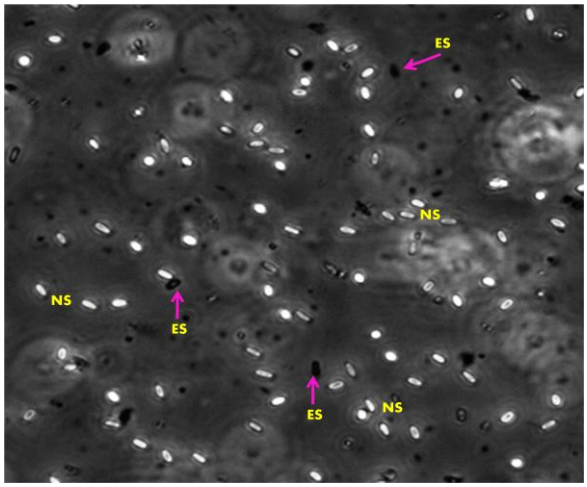


Figure 4.8: Light micrograph of M-Scy spores in water suspension (400X), ES: Empty spore, NS: Non germinated spore

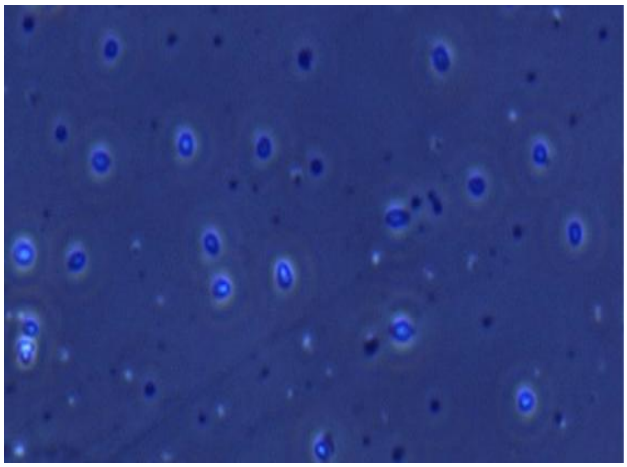


Figure 4.9: Light micrograph of M-Scy spores stained with Nigrosin stain solution (400X)

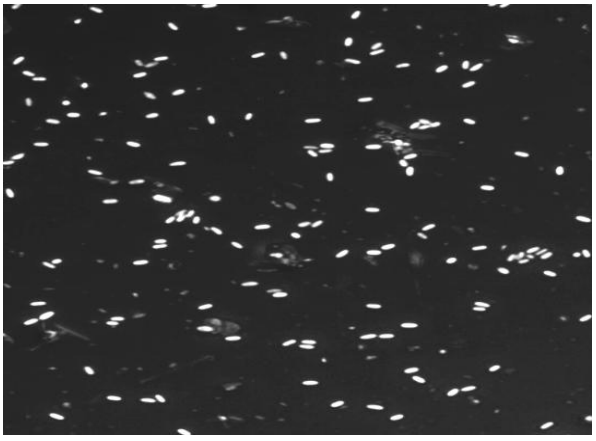


Figure 4.10: Light micrograph of *N. bombycis* spores in water suspension (400X), ES: Empty spore, NS: Non germinated spore

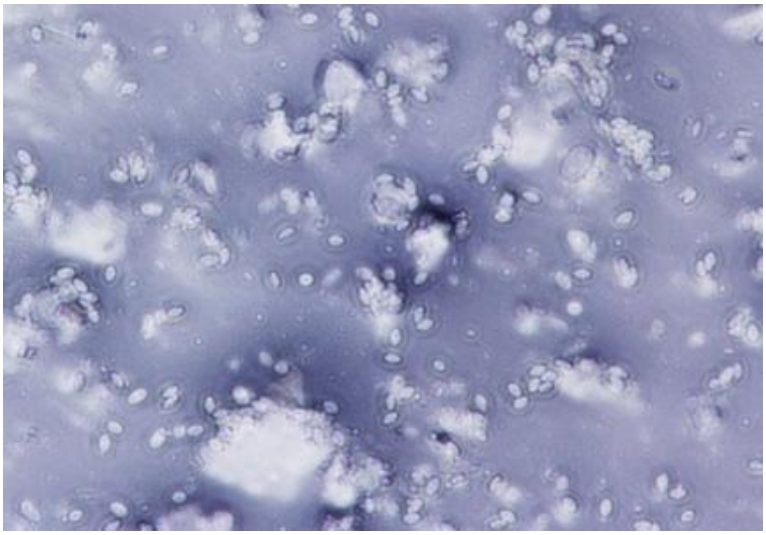


Figure 4.11: Light micrograph of *N. bombycis* spores stained with Nigrosin stain solution (400X)

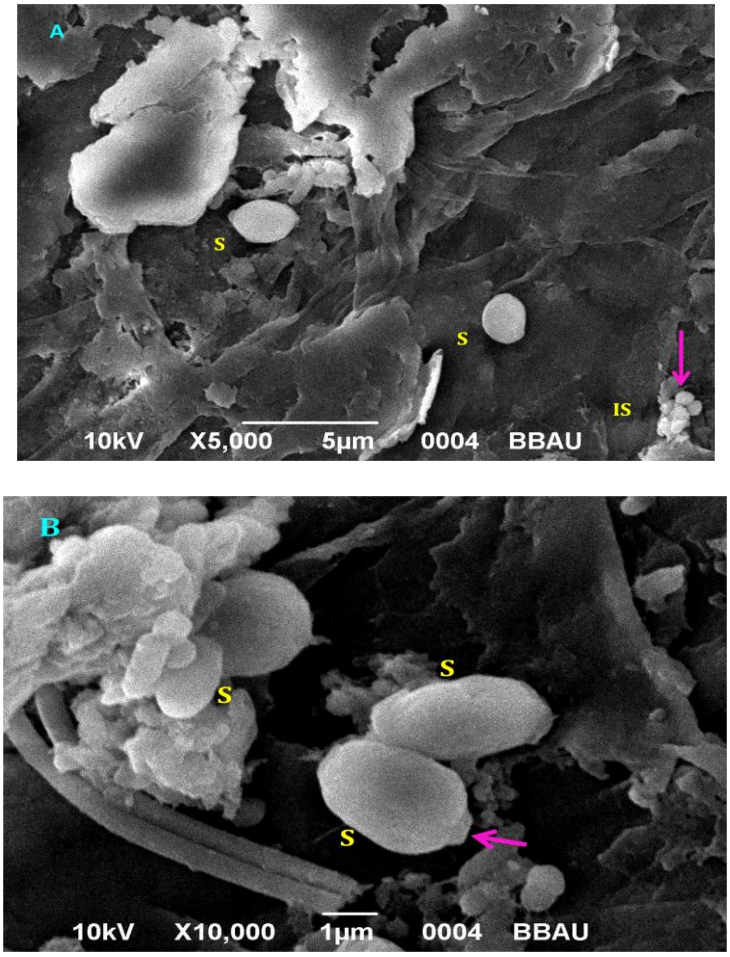


Figure 4.12.: Scanning electron micrograph of M-Dch spores A) Arrow indicates grouping of immature M-Dch spores B) Mature M-Dch spores, Arrow indicates protrusion of spore wall C) A mature M-Dch spore with spore wall protrusion

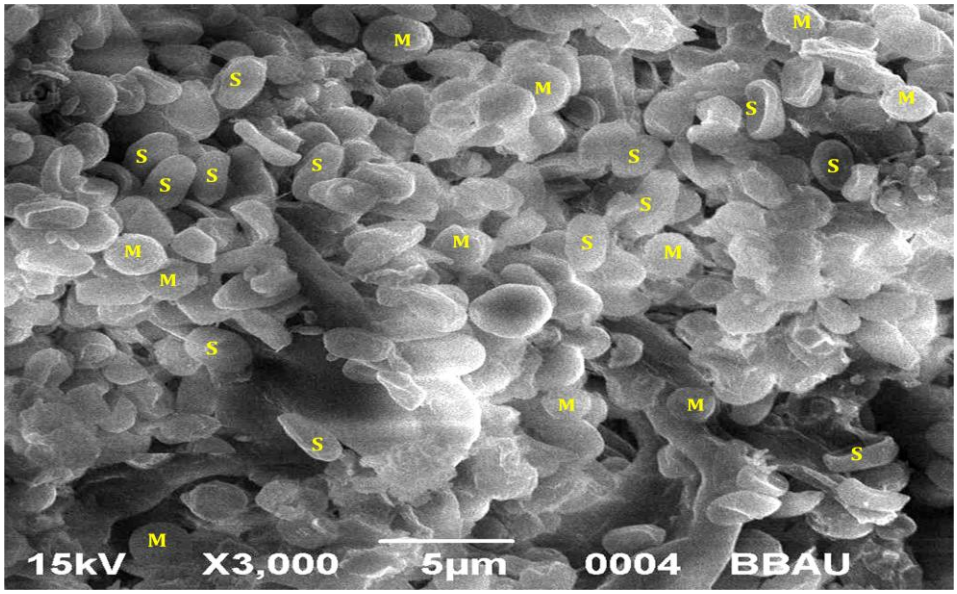


Figure 4.13: Scanning electron micrograph of Gut epithelium cells of *C. florella* filled with M-Cfl spores

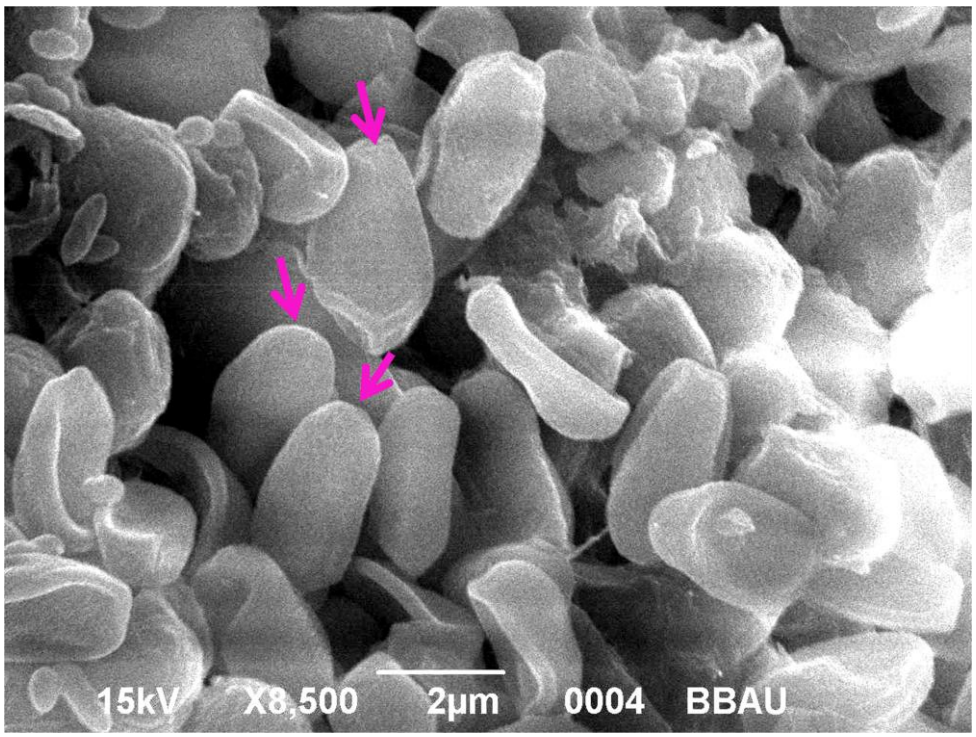


Figure 4.14: Scanning electron micrograph of mature M-Cfl spore (Arrowhead) in the gut epithelium of *C. florella*

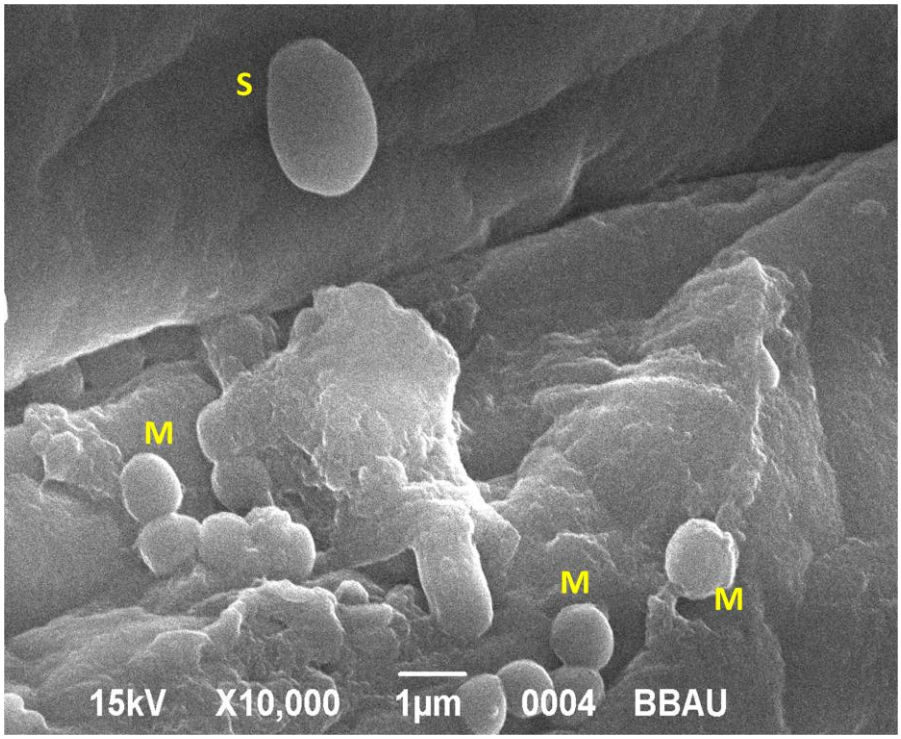


Figure 4.15: Scanning electron micrograph of Gut epithelial cells of *M.phedima* filled with M-Mph spores

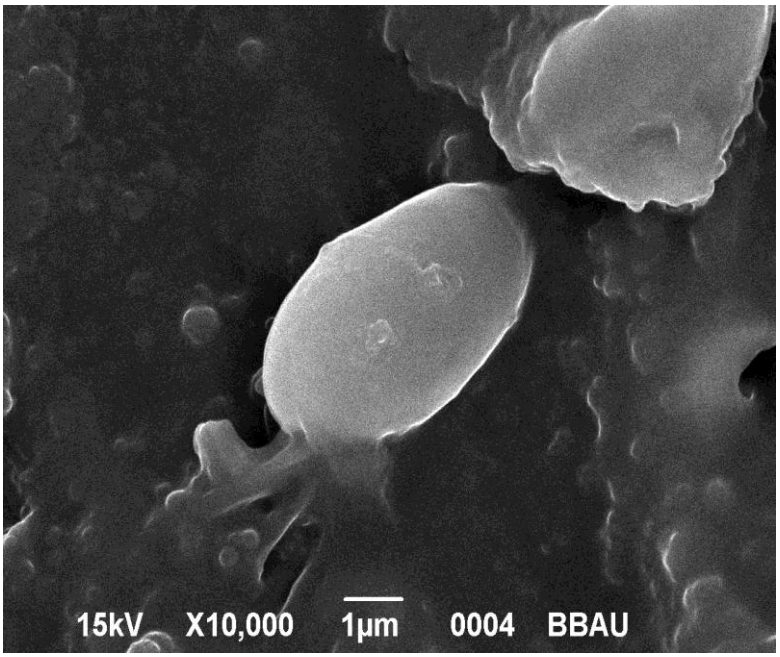


Figure 4.16: Scanning electron micrograph of a mature M-Mph spore in the gut epithelium of *M. phedima*

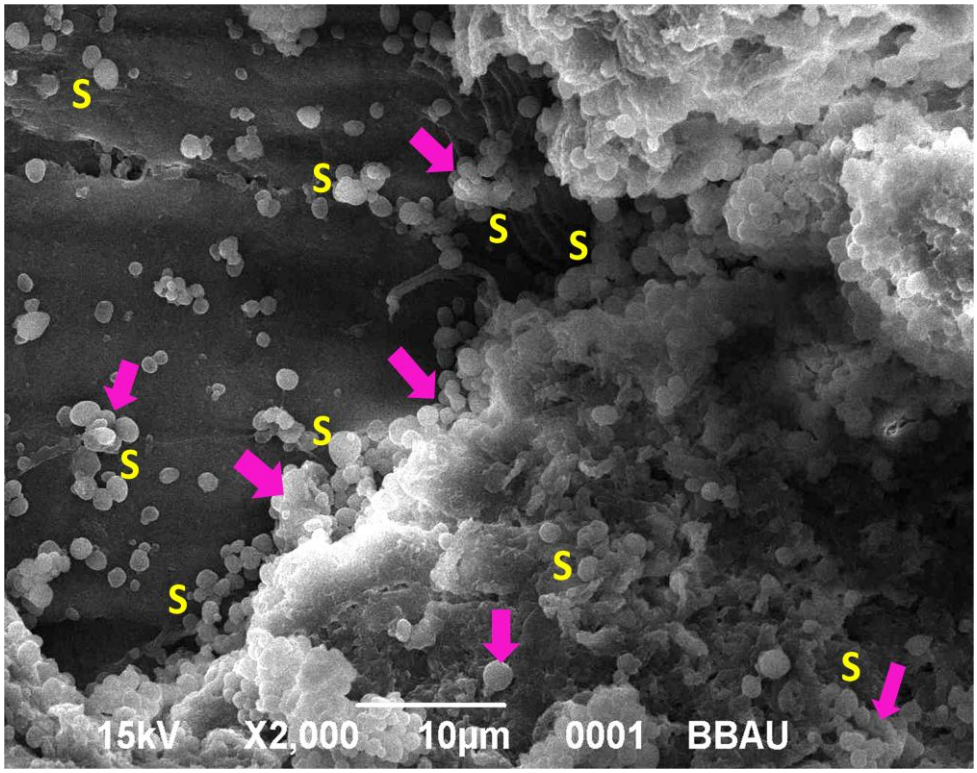


Figure 4.17: Scanning electron micrograph of M-Ame spores in the intestinal epithelial cell of the honeybee, *A. mellifera*

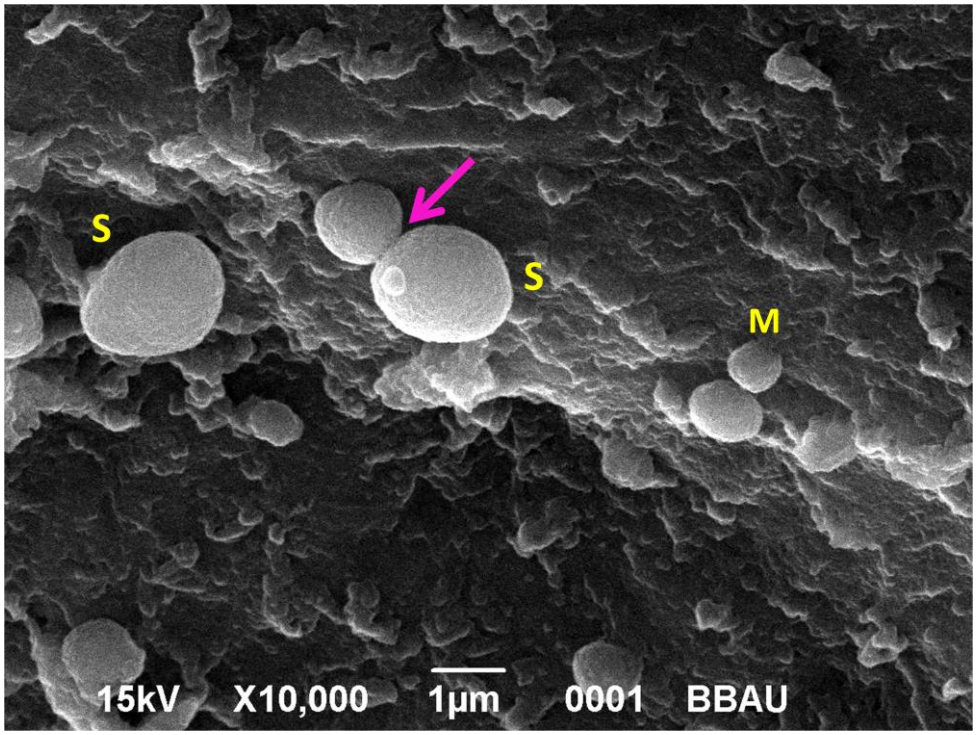


Figure 4.18: Scanning electron micrograph of M-Ame spores undergoing binary fission (Arrowhead)

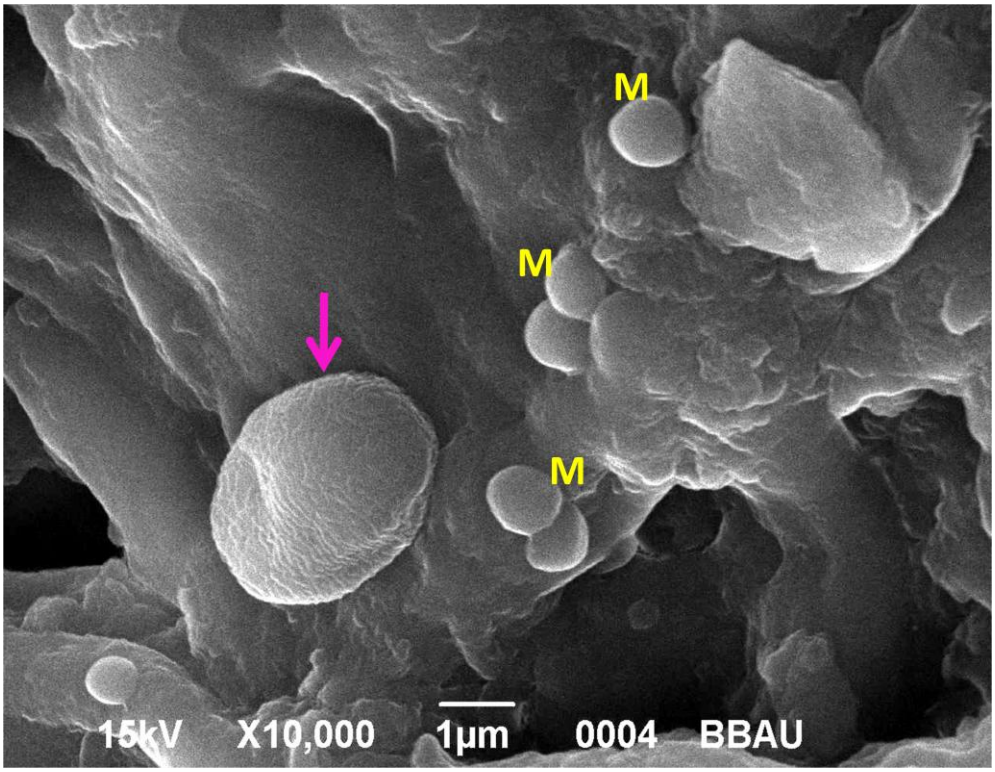


Figure 4.19: Scanning electron micrograph of a mature M-Ame spore (Arrowhead)

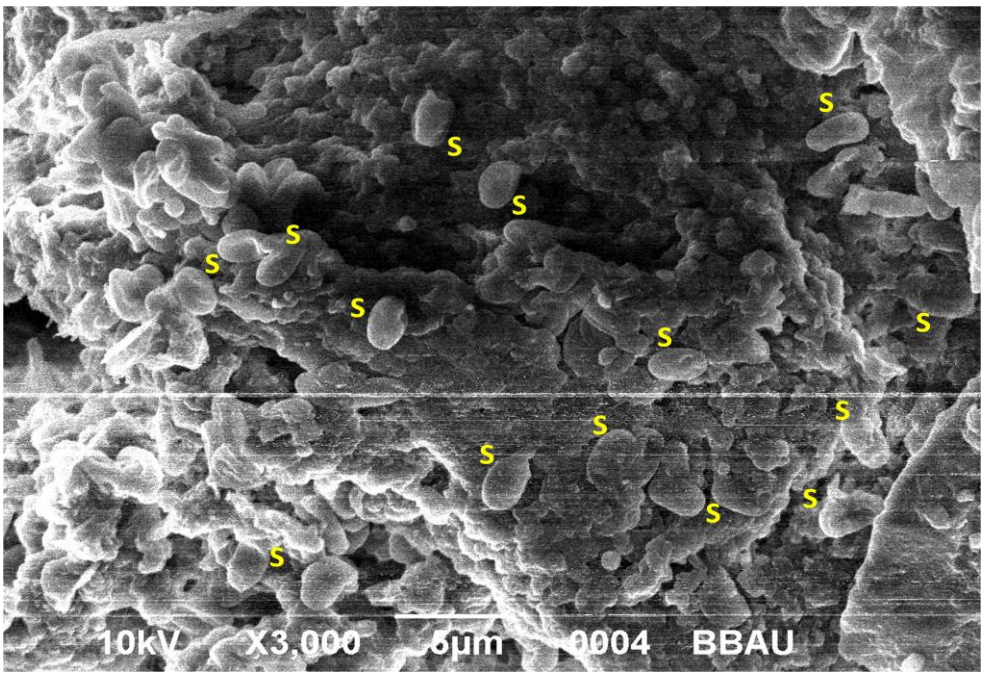


Figure 4.20: Scanning electron micrograph of M-Scy spores in the intestinal epithelial cell of *S. Cynthia ricini*

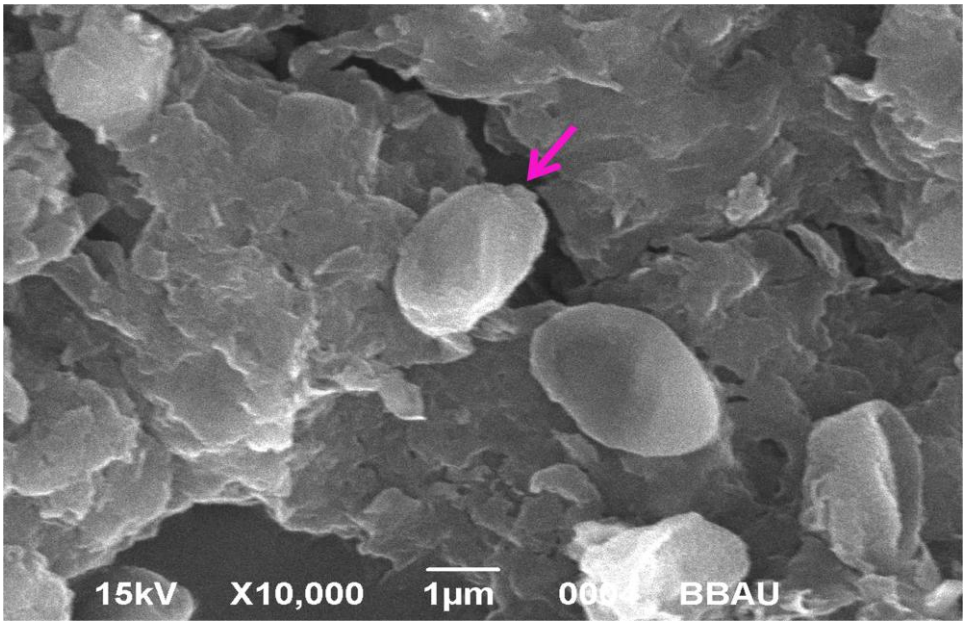


Figure 4.21: Scanning electron micrograph of M-Scy spore showing the Protrusion of spore wall (Arrowhead)

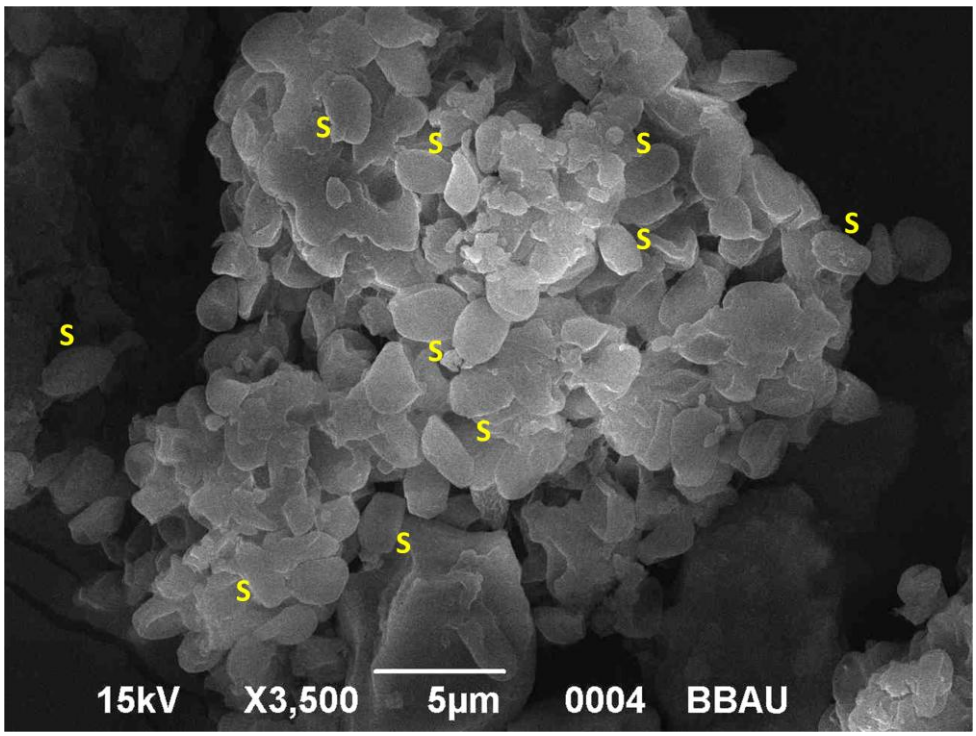


Figure 4.22: Scanning electron micrograph of N. *bombycis* spores in the intestinal epithelial cell of *B. mori*

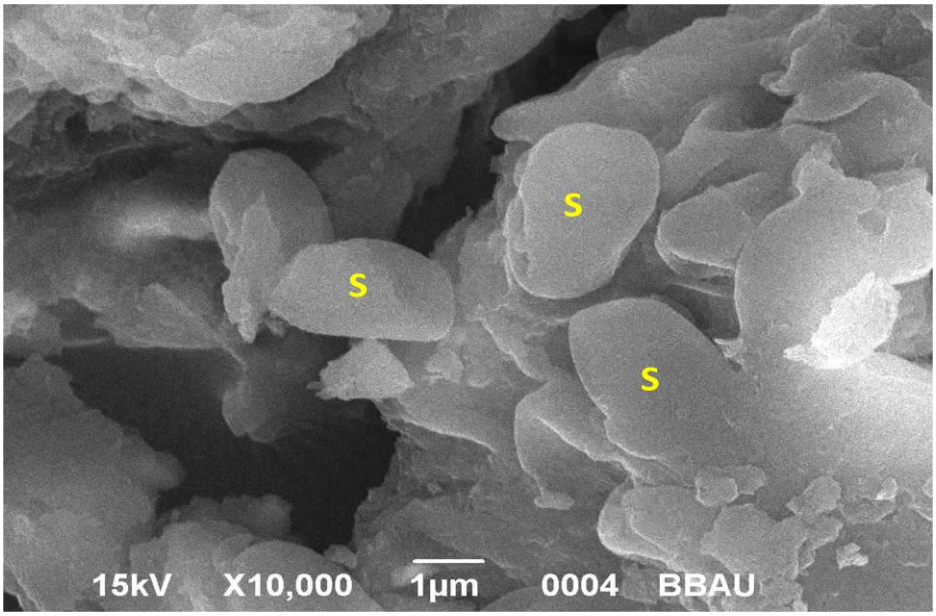


Figure 4.23: Scanning electron micrograph of mature *N. bombycis* spores

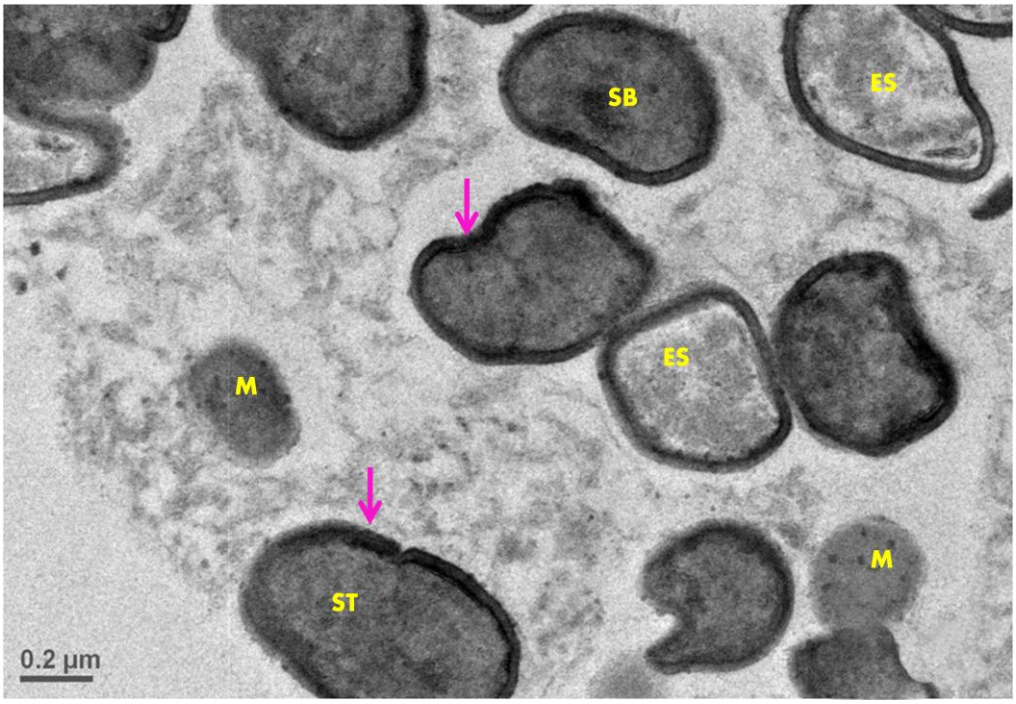


Figure 4.24: Transmission electron micrograph midgut Epithelial cells of *D. chrysippus* infected with different developmental stages of M-Dch spores, M: Meront, ST: Sporont, SB: Sporoblast, ES: Empty spore

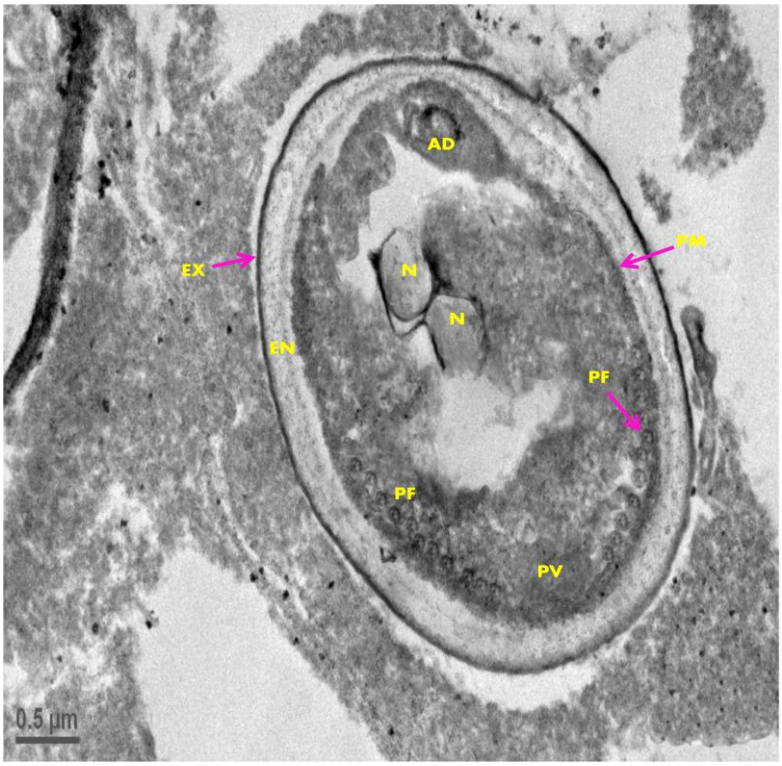


Figure 4.25: Transmission electron micrograph of a mature M-Dch spore showing 11 PF coils, Ex: Exospore, EN: Endospore, PM: Plasma membrane, AD: Adhesive disk, PF: Polar Filament, PV: Posterior vacuole, N: Nucleus

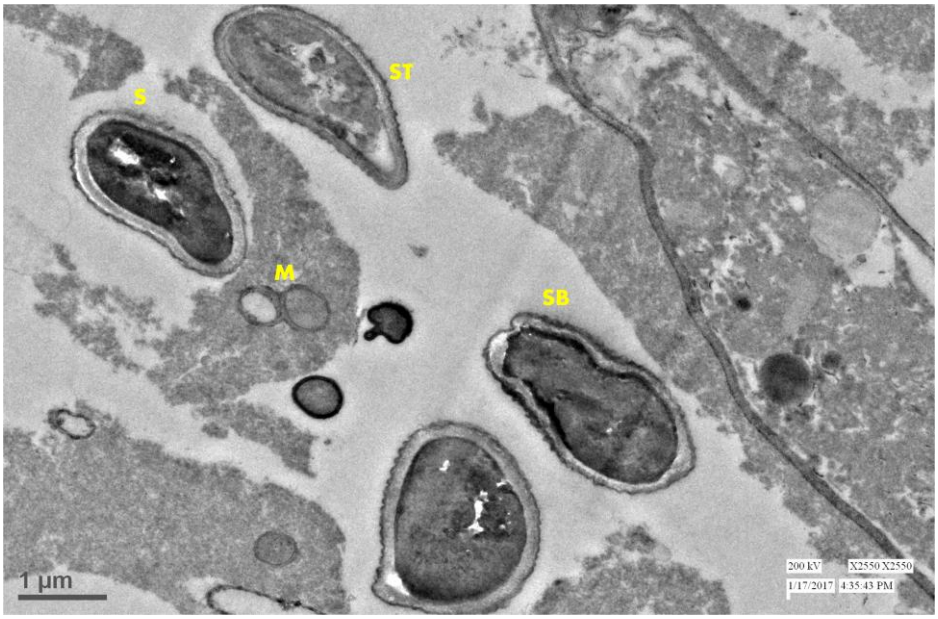


Figure 4.26: Transmission electron micrograph of midgut Epithelial cells of *C. florella* infected with different developmental stages of M-Cfl spores, M: Meront, ST: Sporont, SB: Sporoblast, S: Spore, ES: Empty spore

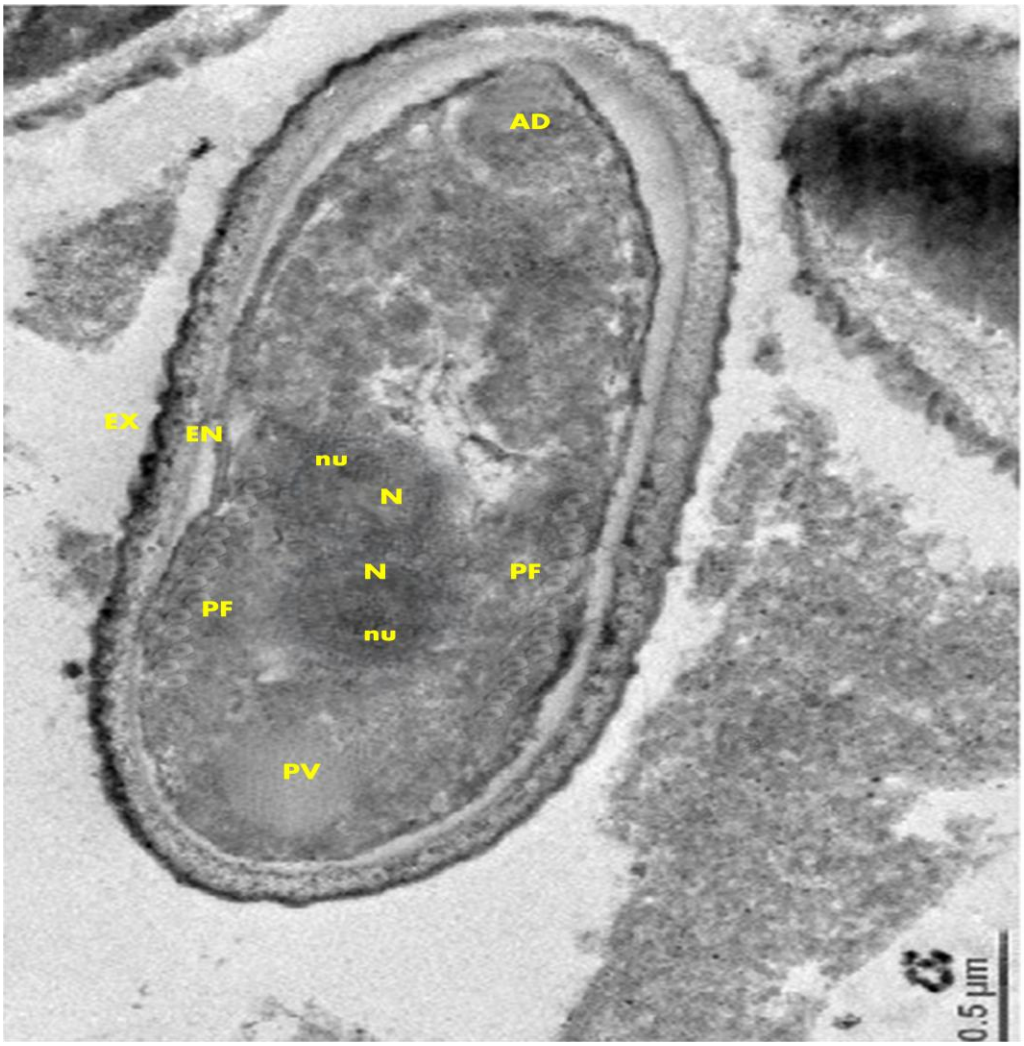


Figure 4.27: Transmission electron micrograph of a mature M-Cfl spore showing 14 PF coils, Ex: Exospore, EN: Endospore, PM: Plasma membrane, AD: Adhesive disk, PF: Polar Filament, PV: Posterior vacuole, N: Nucleus, nu: Nucleolus

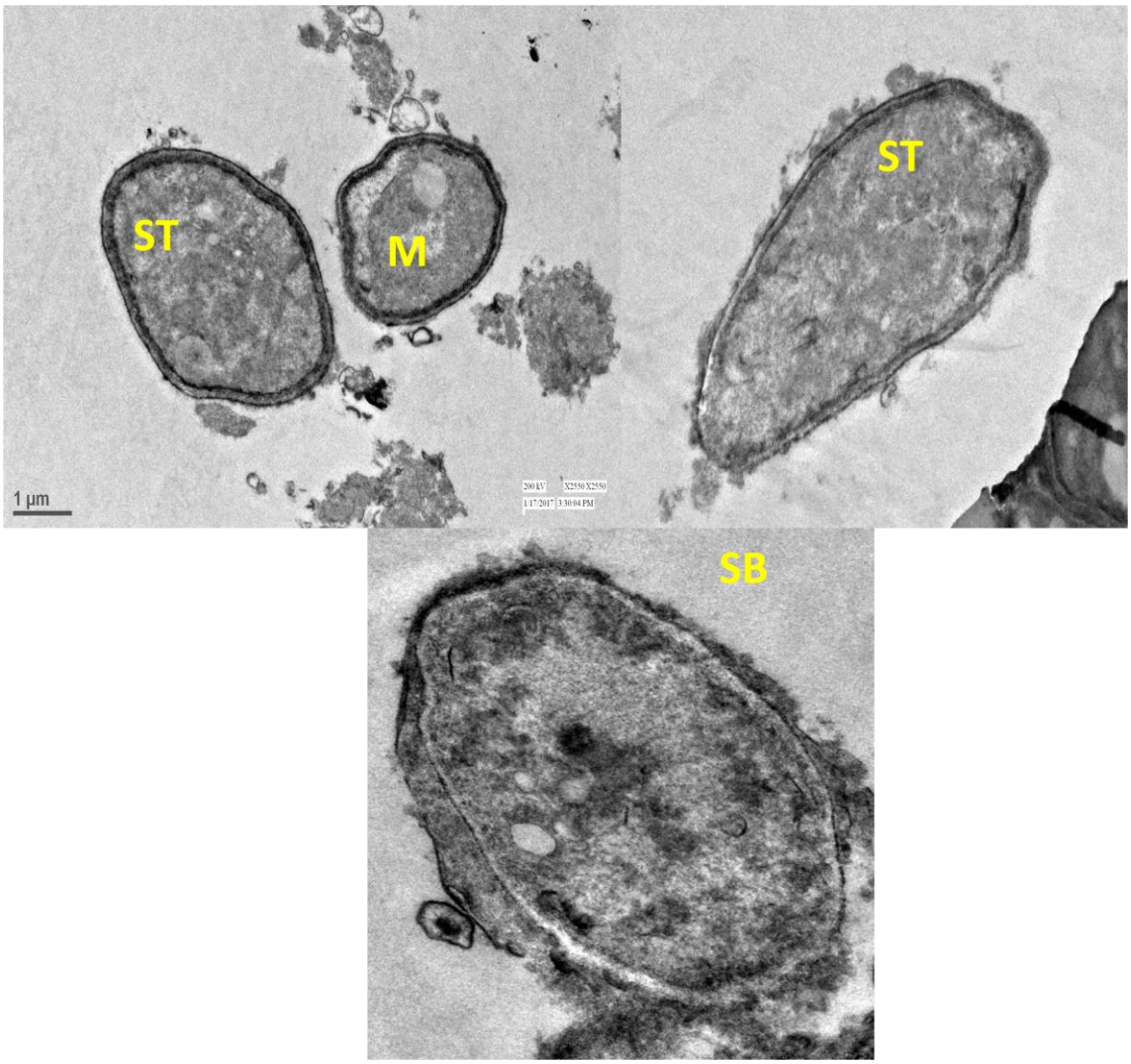


Figure 4.28: Transmission electron micrograph of midgut Epithelial cells of *M.phedima* infected with different developmental stages of M-Mph spores, M: Meront, ST: Sporont, SB: Sporoblast

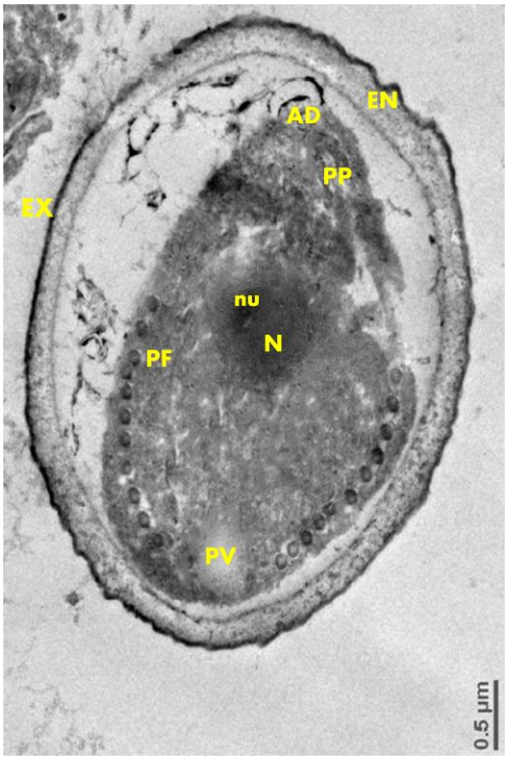


Figure 4.29: Transmission electron micrograph of a mature M-Mph spore showing 11 PF coils, Ex: Exospore, EN: Endospore, PM: Plasma membrane, AD: Adhesive disk, PP: Polaroplast, PF: Polar Filament, PV: Posterior vacuole, N: Nucleus, nu: Nucleolus

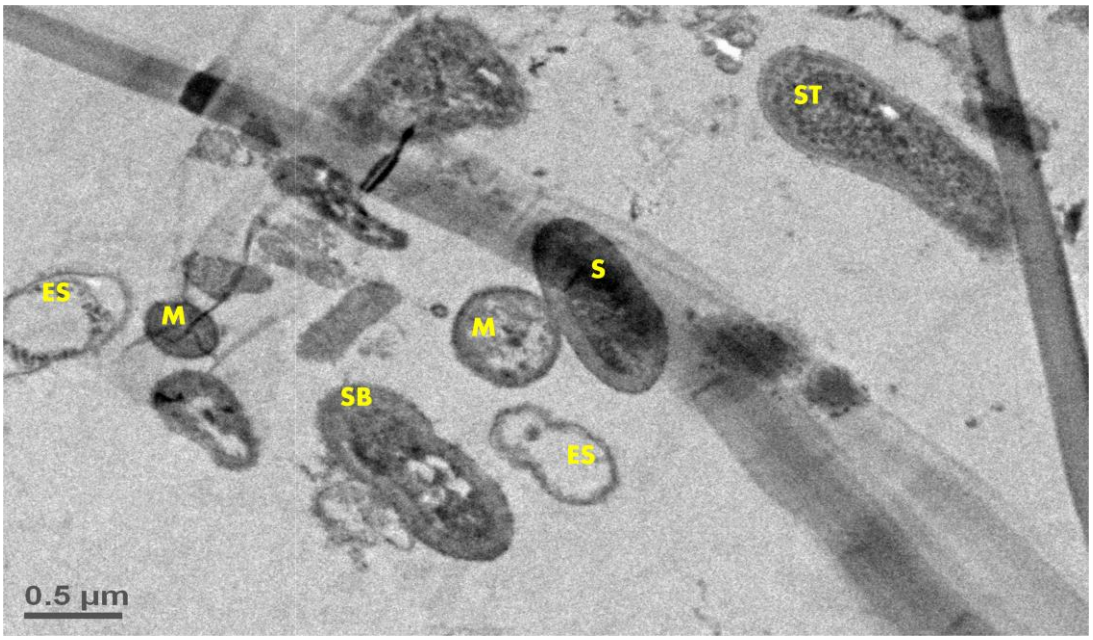


Figure 4.30: Transmission electron micrograph of midgut Epithelial cells of *A. mellifera* infected with different developmental stages of M-Ame spores, M: Meront, ST: Sporont, SB: Sporoblast, S: Spore, ES: Empty spore

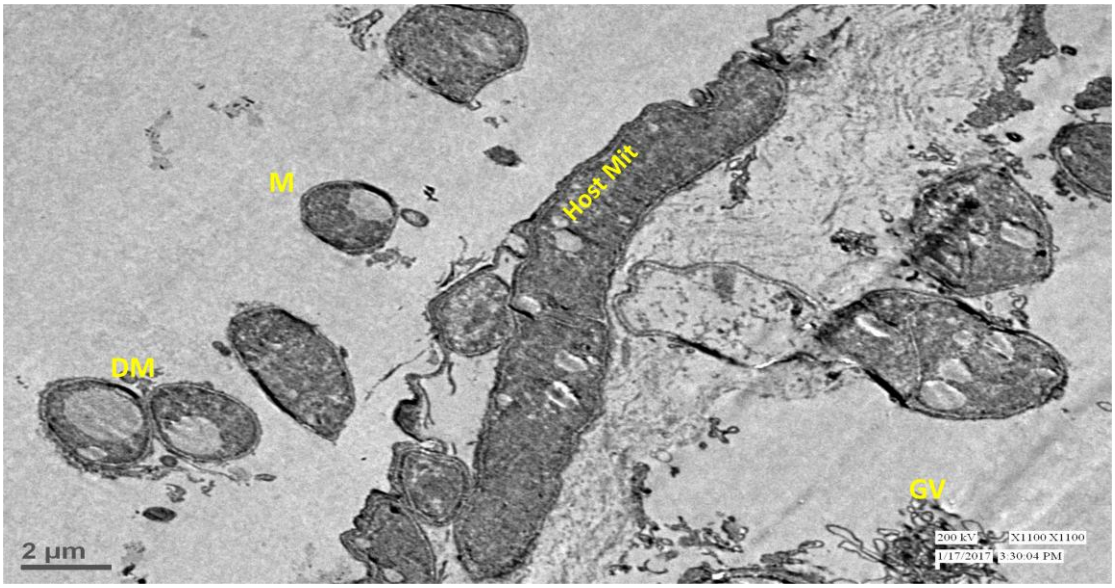


Figure 4.31: Transmission electron micrograph showing midgut Epithelial cells of *A. mellifera* M-Ame spores surrounded by showing Golgi vesicle (GV) and Host mitochondria (Host Mit) in the early merogonial stages

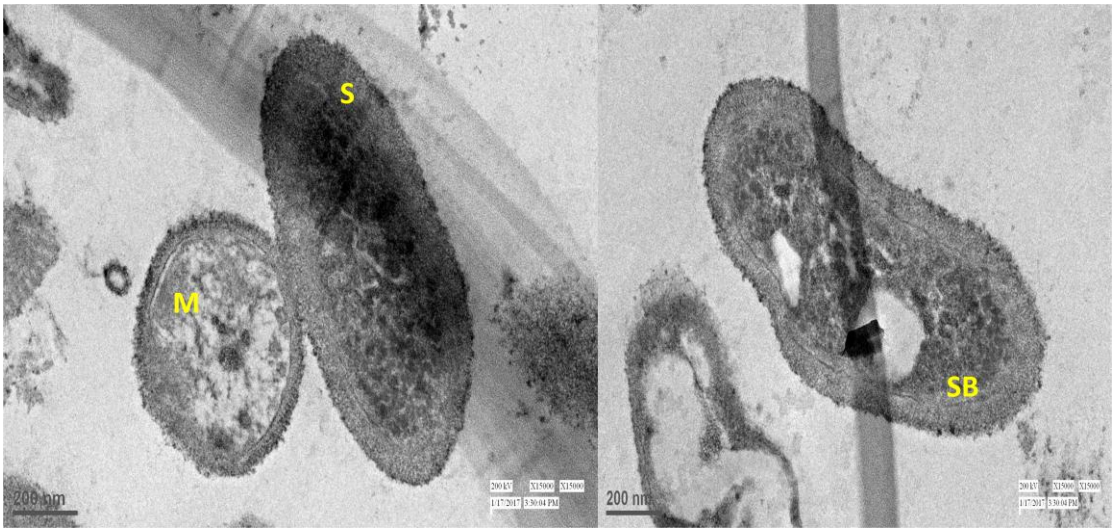


Figure 4.32: Transmission electron micrograph of sporoblast (SB) showing the thickening of spore wall

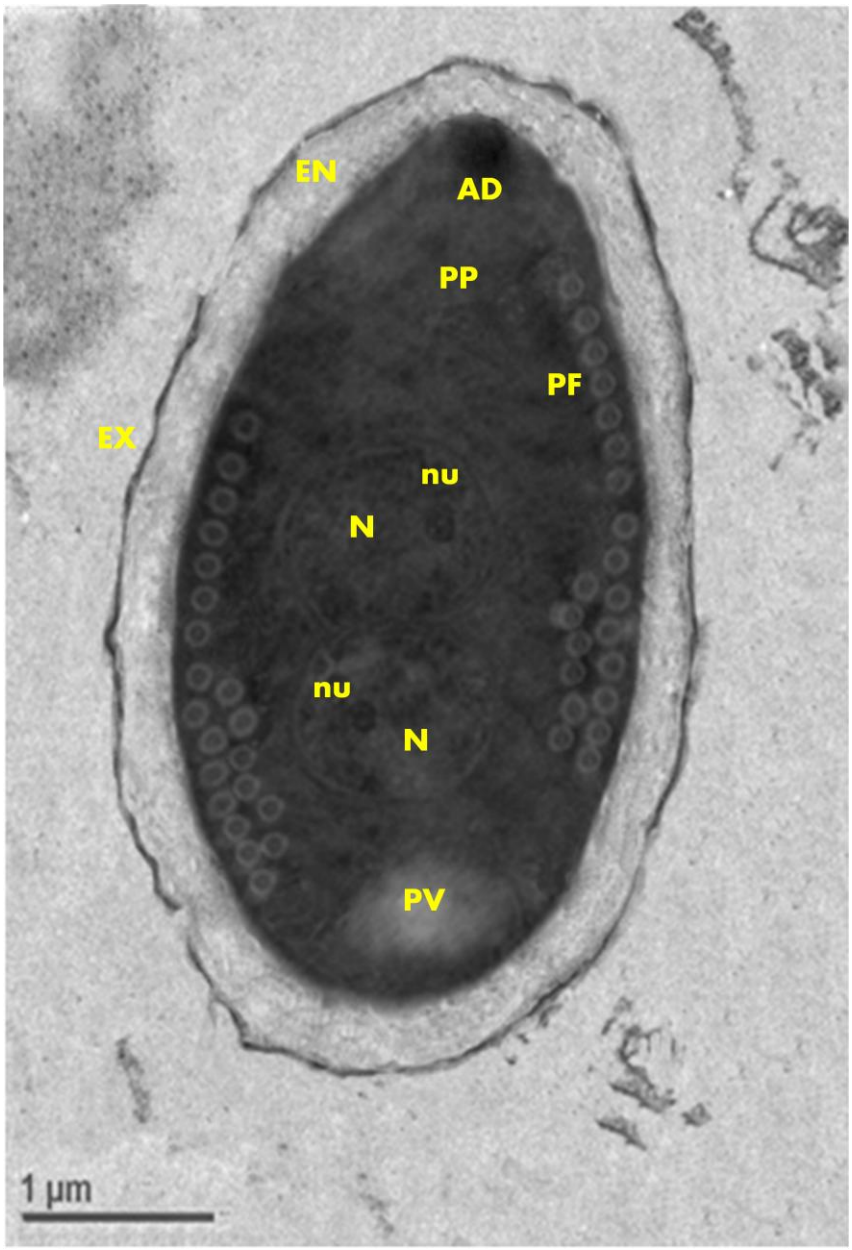


Figure 4.33: Transmission electron micrograph of a mature M-Ame spore showing 21 PF coils, Ex: Exospore, EN: Endospore, PM: Plasma membrane, AD: Adhesive disk, PP: Polaroplast, PF: Polar Filament, PV: Posterior vacuole, N: Nucleus, nu: Nucleolus

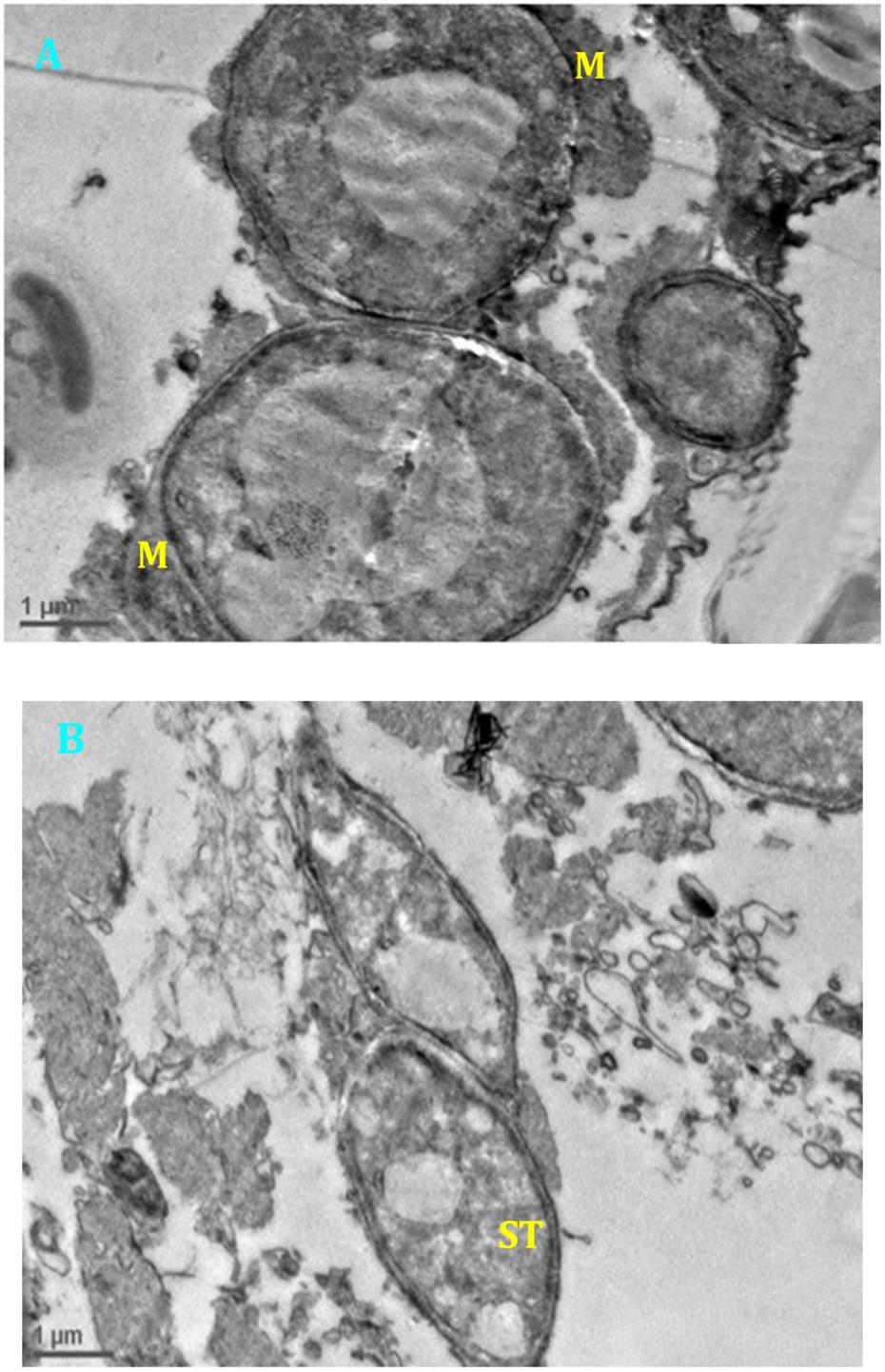


Figure 4.34: Transmission electron micrograph of midgut Epithelial cells of *S. Cynthia ricini* infected with different developmental stages of M-Scy spores, A) Meronts (M) surrounded by a plasma membrame B) Sporont (ST) with some internal structure

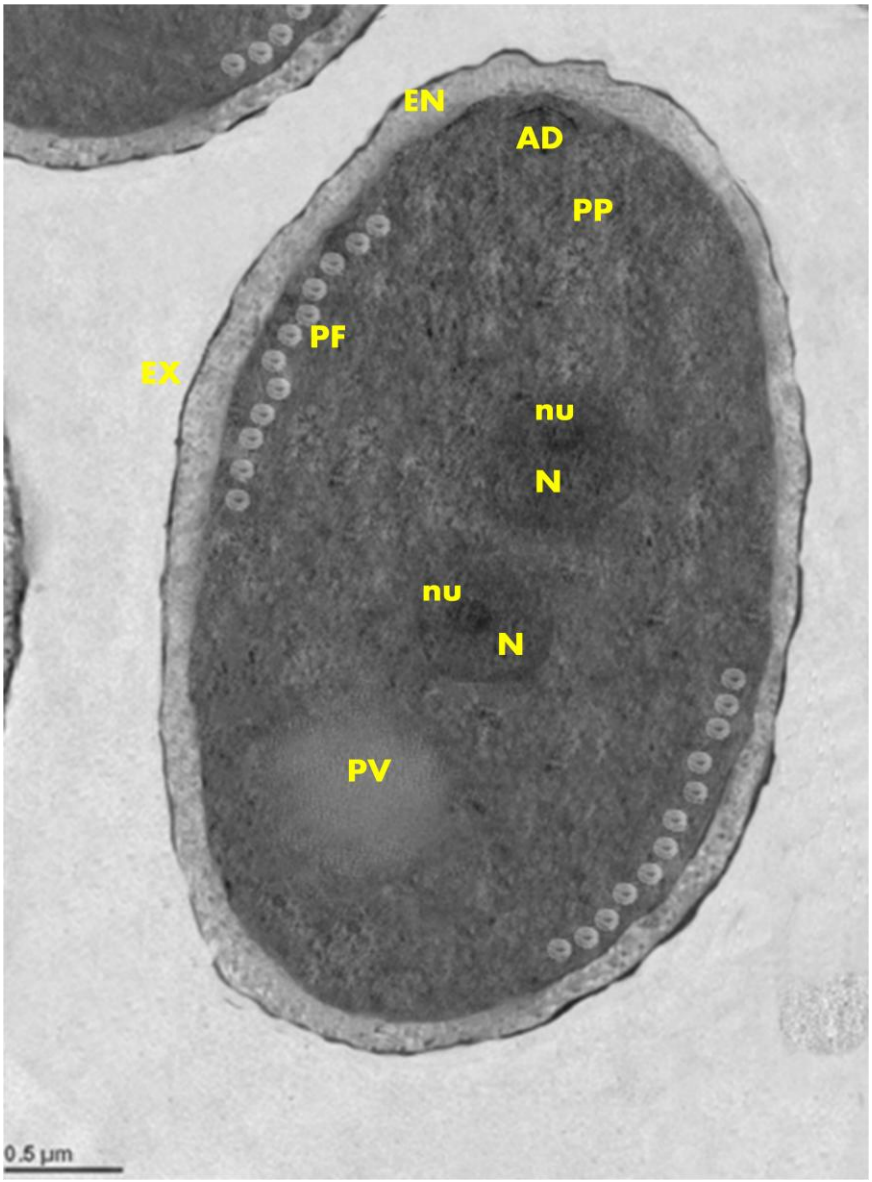


Figure 4.35: Transmission electron micrograph of a mature M-Scy spore showing 12 PF coils, Ex: Exospore, EN: Endospore, PM: Plasma membrane, AD: Adhesive disk, PP: Polaroplast, PF: Polar Filament, PV: Posterior vacuole, N: Nucleus, nu: Nucleolus

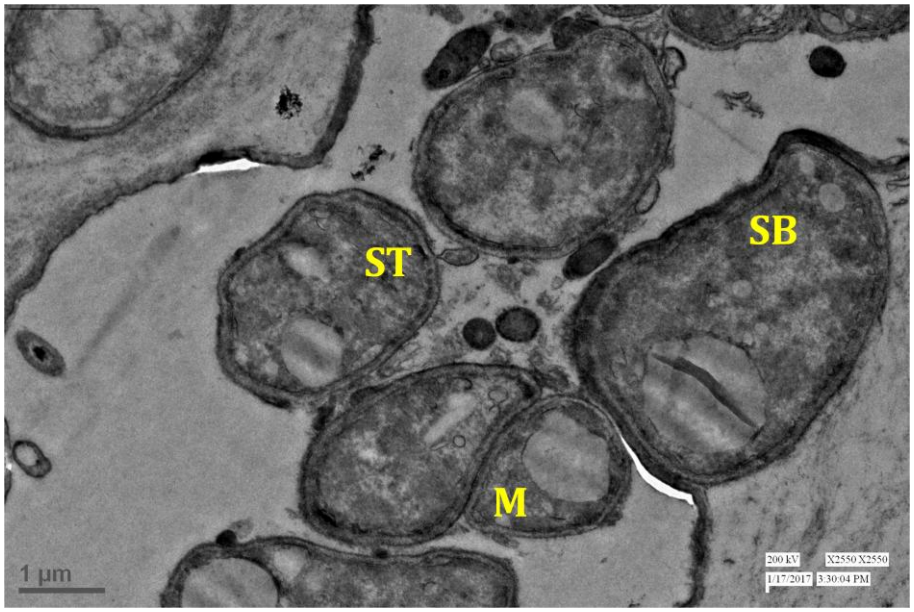


Figure 4.36: Transmission electron micrograph of midgut Epithelial cells of *B. mori* infected with different developmental stages of *N. bombycis* spores, M: Meront, ST: Sporont, SB: Sporoblast

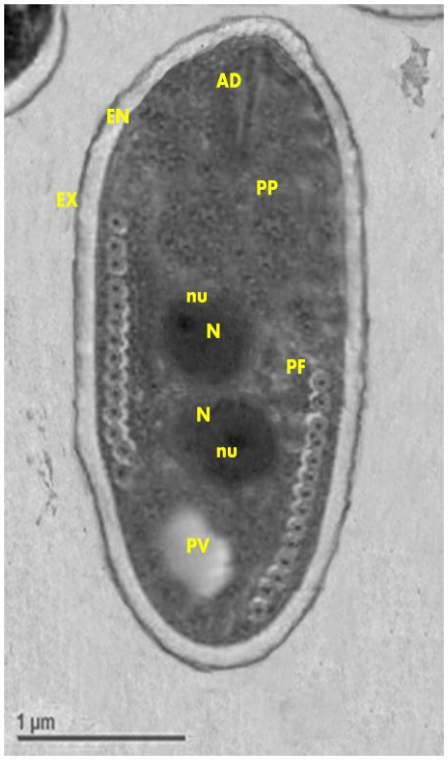


Figure 4.37: Transmission electron micrograph of a mature *N. bombycis* spore showing 14 PF coils, Ex: Exospore, EN: Endospore, PM: Plasma membrane, AD: Adhesive disk, PP: Polaroplast, PF: Polar Filament, PV: Posterior vacuole, N: Nucleus, nu: Nucleolus

4.3 DISCUSSION

The present study is the first documentation of the prevalence of microsporidian infection in the insect species in Lucknow district of Uttar Pradesh. The pathogen found in the insect species *D. chrysippus, C. florella, M. phedima, A.mellifera* and *S. cynthia ricini* are definitely a microsporidium. The microsporidia isolated from the above insect pests were designated as M-Dch, M-Cfl, M-Mph, M-Ame and M-Scy respectively. Further, light microscopic techniques have proved inadequate to differentiate between species of similar appearance; ultrastructural methods have become more and more important (Hazard and Oldacre, 1975; Weiser, 1977). For differentiation of microsporidian species, the attention of taxonomists has been increasingly drawn to the surface structures of the spores.

The study of spore ultrastructure elucidated the typical characteristics such as the presence of polar filament coils, an anchoring disc, polaroplast, posterior vacuole and lack of mitochondria (Canning and Vavra, 2000). The microsporidia under the genus *Nosema* are characterized by the production of spores with walls consisting of an electron dense exospore and an electron lucent endospore, polar filament coils, and diplokaryotic nuclei (Franzen and Müller, 1999; Huang *et al.*, 2007). The light and electron microscopic studies on the spore structure, texture of spore surface, developmental stages and finally the internal structure of the spore confirmed that the microsporidium M-Dch, M-Cfl, M-Ame and M-Scy belong to the genus *Nosema* Naegeli, 1857 whereas the microsporidium M-Mph belongs to the genus *Larssoniella*. Furthermore, the number of polar tube coils, their arrangement and the angle of helical tilt are conserved for each genus of the microsporidia and therefore, these structures provide very effective taxonomic information (Sprague *et al*., 1992; Vavra and Larsson, 1999).

To date about 200 genera of microsporidia have been reported from both vertebrates and invertebrate hosts (Becnel *et al.*, 2014). In insects, 63 different microsporidian genera had been found (Undeen and Vávra, 1997) and the genus *Nosema* is one of the biggest groups, including more than 200 species. Besides this, the genus *Nosema* is frequently a chronic pathogen of both beneficial and harmful insects, being prevalent especially in the order Lepidoptera (Cheung and Wang, 1995). Though the earlier

studies by some researchers reported that the butterflies belonging to the genus *Danaus, Catopsilia, Melanitis* were positive with the microsporidian infections but there was complete lack of studies on their light microscopic structures and ultrastructural details. However, there is availability of structural details of microsporidia infecting the insect species *S. cynthia ricini* and *A. mellifera*.

In the present study the microsporidia M-Dch and M-Cfl were identified as the genus *Nosema* on the basis of their ultrastructural details. The microsporidia belonging to the genus *Nosema* possess two nuclei, 11-14 polar filament coils and the angle of tilt of the polar filament to the spore axis is the identifying feature of the genus (Chakrabarty and Manna, 2006). The M-Dch and M-Cfl spore possessed 11 and 14 polar filament coils respectively. In M-DCh spore, the angle of tilt of the most anterior and most posterior polar filament coil to the spore axis were measured as 42° and 90° whereas in case of M-Cfl spore, the angle of tilt of the most anterior to posterior polar filament coil to the spore axis were measured as 90°. The number of polar coils and their tilting angles of M-Dch and M-Cfl are similar to that of *Nosema* sp. described earlier. *N. mesnili* from *P. brassicae* was reported to have 11-12 coils with the angle tilt being 40-45° (Sokolova *et al.*, 1988) and *Nosema* sp. from *P. rapae* was reported to have 12 coils (Choi *et al.*, 2002). Furthermore, on the basis of ultrastructural studies of the microsporidia infecting the four varieties of silkworm *viz. B. mori, Antheraea myllita, Philosamia ricini and A. assamensis,* they were identified as *Nosema* genus (Chakrabarty and Manna, 2006). The microsporidium *N. myllitta* from *Antheraea myllita* has 12-14 coils with the angle of tilt on most anterior to posterior coil is 90°. The microsporidia *N. bombycis* from *B. mori* had 11-14 coils with the angle of tilt on most anterior to posterior coil is 62° and 61° whereas the microsporidia *N. assamensis* from *A. assamensis* had 9-10 coils with the angle of tilt on most anterior to posterior coil is 50°. The microsporidia isolated from the eri silkworm, *Philosamia ricini* (older name) have been earlier identified as *Nosema ricini* which possesses 10-12 polar filament coils with the angle of tilt on most anterior coil was 42 and to the most posterior coil was 43°. The exospore and endospore of the *N. ricini* ranged from 15-50 nm and <200 nm respectively (Chakrabarty and Manna, 2006; Chakrabarty *et al.*, 2012). In the present study, the M-Scy spore contained 12 polar filament coils with an angle of tilt of the most

anterior to posterior coil to that of the spore axis measured as 40°. Further the exospores and endospore wall of M-Scy spore were measured as 22 nm and 120 nm respectively. In the present study, on the basis of results from the TEM study of the microsporidium M-Scy it was identified as *Nosema* sp. because it matched the entire characteristic features of this genus *Nosema* and particularly to the microsporidium *N. ricini*. However, structures of M-Scy revealed by SEM studies were different from that of *N. ricini*. The SEM study of M-Scy spore showed the spores were oval in shape with a smooth exospore coating whereas the SEM study of *N. ricini* by Chakrabarty *et al.* (2012) revealed that the mature spore had slight concavity at both ends with smooth surface.

The microsporidia known to infect the European honey bee, *A. mellifera* have been identified as *Nosema apis* or *Nosema ceranae* (Higes *et al.,* 2007; Chen *et al.,* 2009). In the present investigation, the microsporidia M-Ame infecting the European honey bee, *A. mellifera* were measured as 4.35±0.10 µm × 2.80±0.07 µm and the TEM study revealed that, the M-Ame spore possessed 21 polar filament coils arranged in two rows with an angle of tilt of 55°. Previous studies on the microsporidian infection in *A. mellifera* by some researchers suggested that, the microsporidium *N. ceranae* contained 20-23 polar filament coils whereas the microsporidium *N. apis* contained more than 30 polar filament coils (Fries *et al.,* 1996). Recently, a new microsporidium *N. neumanni* have been reported from *A. mellifera* in Uganda which is completely different from both *N. ceranae* and *N. apis* and posses 10-12 polar filament coils (Chemurot *et al.,* 2017). Again the size of *N. neumanni* was relatively smaller than the spore size of *N. ceranae* and *N. apis* which measured 2.36±0.14 µm in length and 1.78±0.06 µm in width. In contrast to this, the size of *N. apis* measured as 6.0×3.0 µm (Fries *et al.* 1996) whereas the size of *N. ceranae* measured 4.4±0.41 µm in length and 2.2±0.09 µm in width (Chen *et al.,* 2009). In the present study the average spore size of the M-Ame spore was very close to the average spore size of *N. ceranae.* At the study of ultrastructure level, the M-Ame spore was identified as *Nosema* sp. The species of the microsporidia can be identified only by molecular data (Bollan *et al.*, 2013). The TEM study showed the M-Mph spore possesses a single nucleus in the centre and had 11 polar coils which suggests that it belongs to the genus other than *Nosema*. Till date, only five different microsporidian genus (*Cystosporogenes*,

Larssoniella, *Orthosomella*, *Vairimorpha* and *Nosema*) have been reported from different lepidopteran insect pests (Becnel *et al.*, 2014). The microsporidia belonging to the genus *Cystosporogenes* has 10-12 polar tube coils, an elongated nucleus and the spore were surrounded by an envelope in the host cell (Canning and Curry, 2004). However, under the TEM, the M-Mph spores don't show such an envelope structure. The microsporidia belonging to the genus *Orthosomella* has 6-8 turns of polar tube coils and a single nucleus (Andreadis *et al.*, 1996). The microsporidia belonging to the genus *Larssoniella* has 10-11 polar tube coils and a nucleus (Weiser and David, 1996,1997). The microsporidia belonging to the genus *Vairimorpha* possesses 10-20 polar tube coils and a diplokaryon, however, they differ from the genus *Nosema* in that they possess a octospore stages in their life cycle whereas this stage is absent in the microsporidia belonging to the genus *Nosema* (Medeiros *et al.*, 2004). The M-Mph spore described in the present investigation possessed a single nucleus and 11 polar tube coils with an angle of tilt of the anterior coil as 25° and therefore, shared ultrastructural characteristics similar to that of the *Larssoniella* genus. Further, the vescicular structure was absent in M-Mph The angle of tilt of polar tube coils to the axis of the spore in the genus *Larssoniella* is 21° which confirmed that M-Mph spores belonged to the above genus.

It is interesting to know that the microsporidium *N. ceranae* was known to infect the Asian honey bee, *A. cerana* until 1990s. However, it was first reported in the European honey bee, *A. mellifera*, in Spain in 2006 (Higes *et al.*, 2006). Before 2006, it was thought that *A. mellifera* was infected by only one microsporidian parasite, *N. apis*, a relatively benign pathogen (Bailey, 1955). Recently, another new microsporidium *N. neumanni* has been reported from the European honey bee (Chemurot *et al.*, 2017). The nosemosis diseases in honey bee leads to Colony Collapse Disorder worldwide and causes heavy economic losses. From a study it has been confirmed that this disease spread when an infected bees feed the uninfected bees. Besides this, the younger uninfected bees that were fed by older infected bees developed the *Nosema* infections at a level 13-times higher than young uninfected bees unable to feed from older infected bees (Smith, 2012).

4.4 CONCLUSION

Microsporidian infections in insects are generally chronic, causing pathogenic effect on host and reduce their fecundity and life spans. Microsporidia are highly prevalent in insect population and they are diagnosed under the microscope by their transluscent properties and Brownian movement. However, the shape, size, spore surface and number of polar filament and their angle of tilt differ for each microsporidian species. The SEM study showed that the exospores of M-Dch, M-Cfl, M-Mph and M-Scy were smooth whereas exospores of M-Ame spore was ornamented with ridges and furrows. From the ultrastructure, the microsporidium M-Dch, M-Cfl, M-Ame and M-Scy were identified as genus *Nosema* Naegeli, 1857 whereas the microsporidium M-Mph was identified as genus *Larssoniella*. From the present investigation, it can be concluded that, microsporidian parasites belonging to genus *Nosema* are common in insect pests. The microsporidian infection leads to greater economic losses to a country when they infect the insect species like honey bee, silkworm etc. They are considered to be more useful as long-term regulators of pests and contribute towards the prevention and/or suppression of pest outbreaks. In spite of the role of microsporidia in the natural biocontrol of insect populations, relatively limited informations are available about the diversity of microsporidia. Therefore, the present study provides brief information about the morphology and ultrastructure of the microsporidian parasites harbouring in insect population.

Chapter 5

Studies on the Infectivity of the Microsporidian Spores Isolated from Insect Pests of Mulberry and other Agricultural Crops to the Silkworm Bombyx mori L.

STUDIES ON THE INFECTIVITY OF THE MICROSPORIDIAN SPORES ISOLATED FROM INSECT PESTS OF MULBERRY AND OTHER AGRICULTURAL CROPS TO THE SILKWORM *BOMBYX MORI* L.

5.0 INTRODUCTION

5.0.1 Invention of silk

Silk as a weavable fiber was first discovered by the Chinese empress Hsi Ling Shi during 2640 B.C., one day while sipping tea under a mulberry tree, when a cocoon fell into her cup and began to unravel. However, the culture and weaving was a guarded secret for more than 2,500 years by the Chinese. The Silk industry actually began between 5,000 and 3,000 BC in China and it reached India around 140 AD (Fan and Jin 1993). China and India are the two leading silk producers today. The Silk Road also facilitated the smuggling of silkworm eggs into the Mediterranean region and from there into the rest of Europe. Silk, due to its luster and fine texture, rapidly became a favorite for those people who could afford it. It was the standard for royalty and as gifts for people of stature

5.0.2 Importance of sericulture in developing countries

The art of silk production is called Sericulture and comprises silkworm rearing and post cocoon activities leading to the production of silk yarn. Sericulture is a farm-based, labor intensive and commercially attractive economic activity falling under the cottage and small-scale sector. Sericulture has emerged as an economically viable modern industry and has spread over 60 countries. It is particularly suitable for rural-based farmers, entrepreneurs and artisans as it requires low investment with potential for relatively higher returns. It provides income and employment to the rural poor, especially farmers with small landholdings, and marginalized and weaker sections of the society. Hence, several developing nations like China, India, Brazil, Thailand, Vietnam, Indonesia, Egypt, Iran, Sri Lanka, Philippines, Bangladesh, Nepal, Myanmar, Turkey, Papua New Guinea, Mexico, Uzbekistan and some of the African and Latin American countries have taken up sericulture to provide employment to the people in rural area.

5.0.3 Sericulture Industry in India

India is the second largest producer of raw silk after China and the biggest consumer of raw silk and silk fabrics. India has the unique distinction of being the only country producing all the four known commercial varieties of silk in the world *viz.* mulberry, tasar, eri and muga (Giridhar *et al.*, 2010). Among the four varieties of silk produced in India for the session 2015-16, mulberry raw silk accounted for 71.8% (20,434 MT), tasar raw silk for 9.9% (2,818 MT), eri raw silk for 17.8% (5,054 MT) and muga raw silk for 0.6% (1,66 MT) of the total raw silk production of 28,472 MT (Table 5) (Central Silk Board Report, 2016). The bulk of the commercial silk produced in the world is mulberry silk that comes from the domesticated silkworm, *B. mori* L. which feeds solely on the leaves of the mulberry (*Morus* sp.) plant. In India, mulberry sericulture is confined to the states of Jammu and Kashmir, West Bengal, Karnataka, Andhra Pradesh and Tamil Nadu. Tasar silk is copperish in colour, coarse in nature and mainly used for furnishing and interiors. It is secreted by the tropical tasar silkworm, *Antheraea mylitta* which thrives on Asan and Arjun (*Terminalia* sp.). Rearing is done on naturally growing trees in the forests and is the mainstay for many tribal communities in the states of Jharkhand, Chhattisgarh, Orissa, Maharashtra, West Bengal and Andhra Pradesh. Oak tasar is a finer variety of tasar produced by the temperate tasar silkworm, *Antheraea proylei* which feeds on natural oak plants (*Quercus* sp.) and is found in abundance in the sub-Himalayan belt. Eri silk is a silk spun from open ended cocoons and secreted by the domesticated silkworm, *Samia Cynthia ricini* that feeds mainly on castor leaves. Muga silk is golden yellow in colour primarily produced in the state Assam of India. Muga silk is secreted by *Antheraea assama* that feeds on aromatic leaves of naturally growing Som (*Persia bombycina*) and Sualu (*Litsea polyantha*) plants. In the sericulture industry, production of quality mulberry leaves plays a crucial role in the production of silk. Among the different factors, the mulberry leaf contributes more than one third to the cocoon production. The procedures of mulberry sericulture begin with the cultivation of suitable and best fit mulberry varieties for an eco-zone. Vegetative propagation efficiency is one of the desirable traits of superior varieties

Over the last six decades, the Indian silk industry has registered an impressive growth, both horizontally and vertically. For instance, the age old multivoltine hybrids have been replaced by multivoltine×bivoltine and bivoltine hybrids. The sericulture industry has witnessed a quantum jump in raw silk productivity. The average yield of

25 kgs of cocoons/100 dfls in the recent past has increased and currently the average yields are in the range of 60 -65 kgs/100 dfls (Ganie *et al.*, 2012). In India, Karnataka is the largest producer of silk wherein, 97% of the raw silk is produced in the five Indian states of Karnataka, Andhra Pradesh, Tamil Nadu, West Bengal and Jammu and Kashmir. The state of Uttar Pradesh is at the 12th position in terms of silk production (Table 5.1).

In India, at present, over 60 lakh families, mostly in the States of Karnataka, Andhra Pradesh, Tamil Nadu, West Bengal, Jammu and Kashmir, and Assam are involved in this labour-intensive agro-based industry in activities such as food plant cultivation, silkworm rearing, silk reeling, twisting, de-gumming, weaving, dyeing, printing and finishing, and garment manufacturing. Sericulture is at various stages of development in Uttarakhand, Uttar Pradesh, Madhya Pradesh, Chhattisgarh, Orissa, Bihar, and the North East (Figure 5).

Figure 5: Different types of Silk producing states of India (Source: International Sericulture Commission, 1959)

Table 5: Variety Wise Silk Production in India from during 2012-13 to 2015-16

	Achievements			
	2012-13	**2013-14**	**2014-15**	**2015-16**
Mulberry plantation (Lakh ha)	1.86	2.03	2.20	2.10
Raw Silk Production (MT)				
Mulberry (Bivoltine)	1,984	2,559	3,870	4,613
Mulberry (Cross Breed)	16,731	16,917	17,520	15,865
Sub Total (**Mulberry**)	**18,715**	**19,476**	**21,390**	**20,478**
Vanya Silk (MT)				
Tasar	1729	2,619	2,434	2819
Eri	3116	4,237	4,726	5060
Muga	119	148	158	166
Sub Total (Vanya)	**4964**	**7,004**	**7,318**	**8,045**
GRAND TOTAL	**23,679**	**26,480**	**28,708**	**28,523**

(Source: Central Silk Board, Annual Report, 2016)

Table 5.1: State Wise Raw Silk production in India from 2012-13 to 2015-16

Sl No.	**State**	**Raw Silk Production in MT**			
		2012-13	2013-14	2014-15	2015-16
1	Karnataka	8219	8574	9645	9823
2	Andhra Pradesh	6550	6912	6485	5086
3	Assam & Bodoland	2068	2766	3222	3325
4	West Bengal	2070	2079	2500	2391
5	Jharkhand	1090	2003	1946	2284
6	Tamil Nadu	1185	1120	1602	1898
7	Meghalaya	517	644	656	857
8	Nagaland	324	606	619	631
9	Manipur	418	487	516	522
10	Maharashtra	97	122	221	274
11	Chhattishgarh	391	391	234	261
12	Uttar Pradesh	157	188	236	249
13	Madhya Pradesh	190	195	248	214

14	Jammu & Kashmir	145	136	138	127
15	Odisha	104	53	98	117
16	Telangana			101	116
17	Bihar	22	52	53	67
18	Mizoram	40	44	50	64
19	Tripura	15	40	48	52
20	Arunachal Pradesh	22	15	12	37
21	Himachal Pradesh	23	25	30	32
22	Uttarakhand	17	22	29	30
23	Kerala	6	4	7	9
24	Sikkim	3	0.20	8	6
25	Punjab	5	4	4	0.8
26	Haryana	0.13	0.13	0.3	0.6
	Total	**23,679**	**26,480**	**28,708**	**28,472**

(Source: Central Silk Board, Annual Report, 2016)

5.0.4 Life cycle of mulberry silkworm, *B. mori* L.

Like other lepidopteran insects, silkworm also has four stages in its life cycle *viz.*, egg, caterpillar larva, pupa and adult moth. The silkworm is a cold blooded animal, and therefore, the duration of each life stage completely depends on environmental factors like temperature, humidity, rainfall, light and air. Man interferes this life cycle at the cocoon stage to obtain the silk, a continuous filament of commercial importance, which is used in weaving the fabric. There are three types of mulberry silk cocoons, namely, univoltine, bivoltine and multivoltine. Multivoltine races found in tropical areas have the shortest life cycle with the egg, larval, pupal and adult stages lasting for 9-12 days, 20-24 days, 10-12 days and 3-6 days respectively. In univoltine races, the egg period of the activated egg may last for 11-14 days, the larval period 24-28 days, the pupal period 12-15 days and the adult stage 6-10 days (Table 5.2 and Figure 5.1).

In nature the univoltine races produce only one generation during the spring, and the second generation egg goes through a period of rest or hibernation till the next spring. In the case of the bivoltine races, however, the second generation egg does not

hibernate and hatches within 11-12 days and produces the second generation normally during summer. But it is the third generation egg which undergoes hibernation and hatches only the next spring, thus producing only two generations in a year (bivoltine). In multivoltine races, the life cycle is the shortest because of the warmer ecological conditions where they are reared, and so they may yield as many as seven to eight generations in a year in tropical sericultural areas such as India, Thailand, etc. Silkworm rearing is, therefore, continuous in tropical areas whereas in sub-tropical and temperate zones it is mostly seasonal lasting from spring to early autumn.

Systematic Classification

Phylum- Arthropoda
Class- Insecta
Sub-Class- Pterygota
Order- Lepidoptera
Super Family- Bombycoidae
Family- Bombycidae
Genus- *Bombyx*
Species- *mori*

5.0.4.1 Caterpillar larva

The larval life of mulberry silkworm may last from 20-24 days in the case of the multivoltine species in tropical areas or 24-28 days in the case of uni and bivoltine races in temperate areas, being shorter under warmer summer and autumn conditions, and somewhat longer under cooler spring conditions. During the larval life, the worm moults or casts off its skin four times to be able to grow. In view of the four intervening moults, the larval life is divided into five distinct stages or instars which are referred to popularly as five different ages. The first three instars are referred to as "young ages" and the fourth and fifth instars as "late ages".

5.0.4.2 Moulting

Each larval instar can be broadly divided into two phases: the feeding phase and the moulting phase. After feeding voraciously and having attained full growth for

the particular instar the worm loses its appetite and the larva prepares to moult and cast off its old skin. Prior to each moulting, the larva stops feeding and rests with its head held up. The lustrous body skin gradually becomes translucent, loose and wrinkled and the worm becomes dull in appearance and wanders about in search of a resting place. It emits a silky substance with which it fixes itself to dry leaves.

The moulting periods last for 15-30 hours: being shortest in the second moult, followed by the first, third and fourth respectively. This resting for moulting is often referred to as "going to sleep" and the coming out of the worm from the moult as "waking up". During the moulting period the worms should not be disturbed so that the process of moulting is not interfered and uniform moulting is ensured.

Table 5.2: Duration of different instars and moulting periods (R.H.: Relative Humidity)

	Multivoltine Races		**Uni and Bivoltine Races**	
	Duration	Temperature and Humidity Conditions	Duration	Temperature and Humidity Conditions
I instar	3 days		3 days	27° C and 85% R.H.
I moult	20 hours		20 hours	
II instar	2 days	27° C	2 days	
II moult	20 hours	80-85% R.H.	20 hours	
III instar	3 days		3 days	25° C and 30% R.H.
III moult	1 day		1 day	
IV instar	4 days	25-26° C	5 days	22-24° C and 75% R.H.
IV moult	1 day	70-80% R.H.	1 day	
V instar	6-7 days		9-10 days	20-23° C and 70% R.H.
Total Duration	**22-23 days**		**26-27 days**	

Table 5.3: Characteristics of the breeds/hybrids selected for studies on their susceptibility to the isolated microsporidia

Silkworm Breed/Hybrid	**Characteristics**
Multivoltine Breeds	
Pure Mysore	Local breed of South India
Nistari	Local breed of Eastern India
ND7	Newly developed, highly productive multivoltine breed and component of Jayalaxmi hybrid; under National level race authorization test.
NP1	Newly developed robust multivoltine breed which is in pipeline and subjected to race authorization.
Bivoltine Breed	
CSR2	Productive bivoltine breed and an authorized component of popular hybrid CSR2×CSR4.
CSR4	Productive bivoltine breed and an authorized component of popular hybrid CSR2×CSR4.
CSR18	Authorised robust bivoltine breed, high temperature tolerant and component of CSR18×CSR19.
CSR19	Authorised robust bivoltine breed, high temperature tolerant and component of CSR18×CSR19.
Hybrids (Cross Breeds)	
PM×CSR2	Popular multi×bi hybrid.
CSR2×CSR4	Popular bi×bi hybrid.

5.0.4.3 Mature worms

After the silkworm passes through four moults, it reaches the fifth and final instar when it attains its maximum weight a day prior to maturity and before it stops feeding. At its maximum weight it is about 10000 times its own weight at the time of hatching and this phenomenal growth takes place within the short span of 20-25 days. When the worm is fully mature for mounting, it loses it appetite, stops feeding, and excretes soft faeces with high moisture content. At this stage because of its

voluminous increase in size, the silk gland is visible through the body integument and, therefore, the thorax and body segments of the mature larva appear translucent. In fact the silk glands are so enlarged as to account for nearly 40% of the body itself. This is characteristic of the ripened worm and serves as guidance for picking the mature worms for mounting. The mature worms become very restless and raise their heads in search of support so as to be able to start spinning.

5.0.4.4 Spinning of the cocoon

The spinning of the cocoon starts almost immediately after mounting and in 48-72 hours spinning is completed by the mature worm. In another day or two the worm transforms itself into the pupa within the cocoon. The pupal period may last for 8-14 days after which the adult moth emerges slitting through the pupal skin, and piercing the fibrous cocoon shell with the aid of the alkaline salivary secretion that softens the tough cocoon shell.

The adult moths are ready to copulate immediately after emerging from the pupa and the female then lays the eggs. Adult life is short, lasting from 3-10 days depending upon races and seasons. The adults do not feed and are also incapable of flight. The females are larger in size and generally sluggish while the males are somewhat smaller and more active. A female of the multivoltine variety may lay an average of approximately 400 eggs while the uni-and bivoltine varieties of silkworm moths lay 500-600 eggs.

5.0.5 Mulberry silk

There are four types of natural silk which are commercially known and produced in the world. Among them mulberry silk solely contributes around 90 percent of the world silk production. Therefore, the term "silk" in general refers to the silk produced by mulberry silkworm. Silk also referred as "Queen of Textiles" which spells luxury, elegance, class and comfort. The silk accounts for only 0.2% of world's total textile production (Central Silk Board, Annual Report, 2016) however, the silk production is regarded as an important tool for economic development of a country as it is a labour intensive and high income generating industry that churns out value added products of economic importance.

Geographically, Asia is the main producer of silk in the world and produces over 95% of the total global output. Though there are over 40 countries on the world map of silk, the bulk of it is produced in China and India, followed by Japan, Brazil and Korea. China is the largest supplier of silk to the world and India is the second largest producer of silk (Central Silk Board, Annual Report, 2016). The mulberry silk comes from the silkworm, *B. mori* L. which solely feeds on the leaves of the mulberry plant. These silkworms are completely domesticated and reared indoors. In India, mulberry silk is produced mainly in the states of Karnataka, Andhra Pradesh, Tamil Nadu, Jammu & Kashmir and West Bengal which together accounts for 92% of country's total mulberry raw silk production, while the non-mulberry silks are produced in Jharkhand, Chattisgarh, Orissa and north-eastern states.

5.0.5.1 Composition of silk

The silk cocoon is spun around the contracted body of the pupa which uses silk strands secreted from labial glands analogous to salivary glands in other larval insects (Asakura *et al.*, 2007). Silk strands are polypeptide polymers composed of multiple components *viz.* microfilaments of insoluble proteins (fibroin), covered with a soluble adhesive protein (sericin) which provides structural support for the cocoon (Zhou *et al.*, 2000; Hakimi *et al.*, 2006). Other minor components include small proteins, lipids, and carbohydrates (Gauthier *et al.*, 2004).

5.0.5.2 Medicinal properties of silkworm *B. mori* L.

Despite the production of silk, the silkworm *B. mori* L. exhibits a number of medicinal properties (Fenemore and Prakash, 1992). Some of the properties are as follows:

- The silkworm larvae have a high medicinal value and usually used to reduce blood pressure, diabetes, nerve disorders and heart problems.
- The pupae are being employed to extract vitamins A, B12, E and K
- The male moths are used for making medicinal vines
- The excreta of silkworm forms an important part of fish and poultry feed.

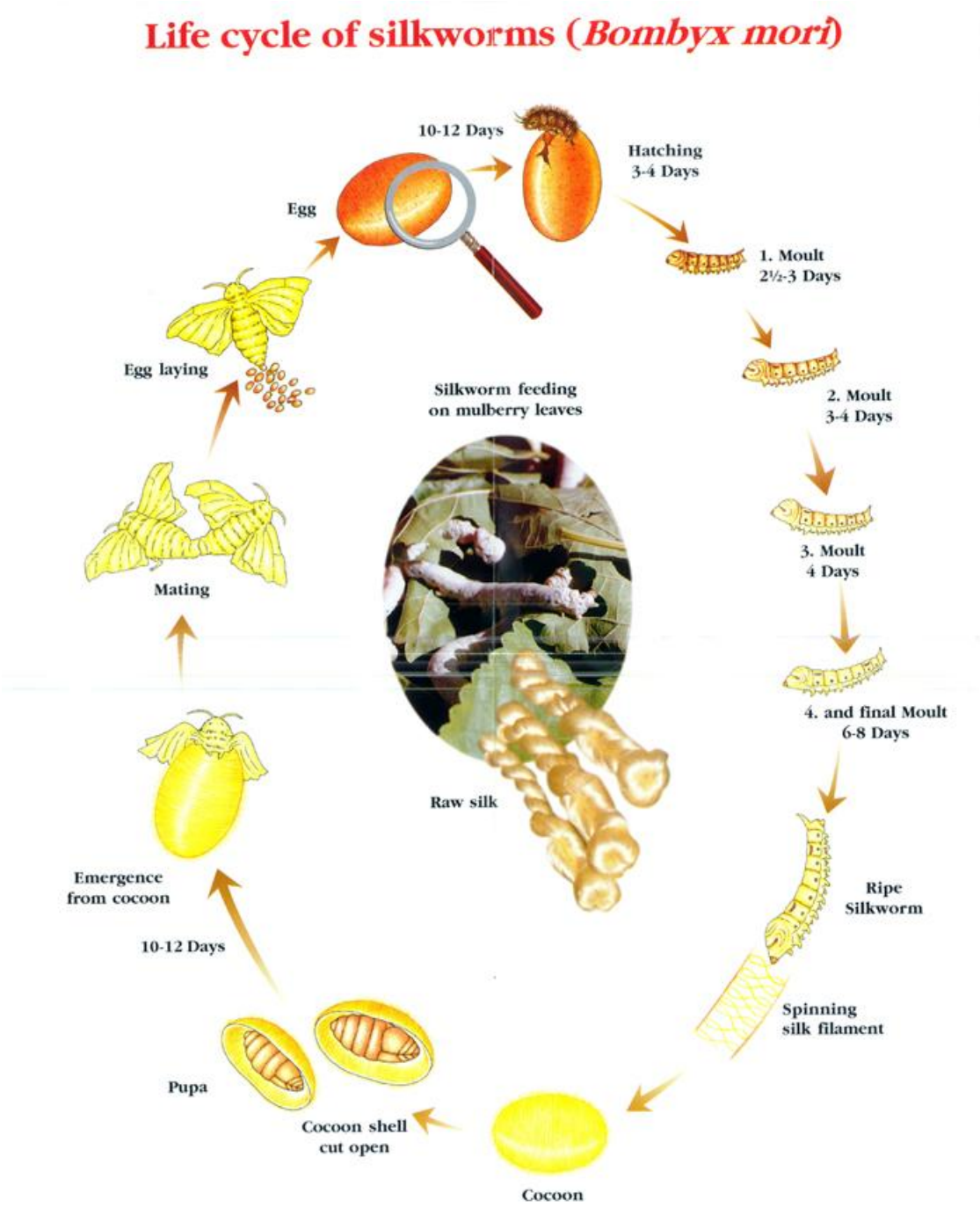


Figure 5.1: Diagram depicting Life cycle of mulberry silkworm, *B. mori* L

5.0.6 Insects inhabiting the Mulberry Plants

Mulberry foliage is the sole food of the silkworm, *B. mori* L. and is grown under various climatic conditions ranging from temperate to tropical climate. The plant is of perennial nature and harbours several insect pests throughout the year. More than 300 insect pests belonging to various orders such as Lepidoptera, Orthoptera, Hemiptera, Coleoptera,

Isoptera and Thysanoptera are known to attack mulberry and cause damage to the crops (Kotikal and Devaiah, 1987). These insect pests are reported to cause heavy economical loss to sericulture farmers and silk industry as the rate of silk production is directly proportional to the quality of leaves used. The average incidence and loss in mulberry leaf yield caused by these pests is estimated to be 34.24% and 4500 kg/ha/yr depending upon the pest and severity (Manjunath, 2004). Further, the production of quality mulberry leaves is necessary for the sericulture farmers in order to ensure healthy growth of silkworms and better silk thread harvest. Some researchers have reported that the perpetual incidence of microsporidian infection in silkworms may be due to various sources of secondary contamination including alternative hosts in and around mulberry fields (Kishore *et al*., 1994; Sharma *et al.*, 2008; Bhuvaneswari and Surendra, 2015a, 2015b). Presumably, insect faeces and scales containing the microsporidian spores might stick to mulberry leaves and transmit the microsporidian infection to silkworm larvae (Kawarabata, 2003). Again, the feeding of silkworm with such contaminated mulberry leaves by various insect pests cause low development and increase larval mortality and consequently there must be a drop in the production of silkworm cocoons which ultimately affects the economy of the country.

Table 5.4: Infectious diseases of the silkworm *B. mori*, L.

Diseases	**Causative agent**	**Symptoms**
Virosis		
Nuclear polyhedrosis	*B. mori* nuclear polyhedrosis virus (*Bm*NPV)	Swollen intersegmental region; shining and fragile skin; milky white fluid.
Cytoplasmic polyhedrosis	*B. mori* cytoplasmic polyhedrosis virus (*Bm*CPV)	Translucent cephalothorax region; diarrhea; retarded growth; milky white midgut; whitish faeces.
Infectious flacherie	*B. mori* infectious flacherie virus (*Bm*IFV)	Translucent cephalothorax; retarded growth; vomiting and diarrhoea.
Densonucleosis	*B. mori* densonucleosis virus (*Bm*DNV)	Translucent cephalothorax; retarded growth; vomiting and diarrhoea.

Mycosis		
White muscardine	*Beauveria bassiana*	Oily specks on the body surface; larva on death softens, turns hard and latter mummifies; mummified larvae appear white.
Green muscardine	*Spicaria prasina* (*Nomuraea riyeli*), *Metarhizium anisopliae*	Large specks with black periphery; mummified larvae green in color
Yellow muscardine	*Pacilomyces farinosus*	Large disease specks around stigma and small on skin, mummified larvae yellow
Red muscardine	*Sporosporella urella*	Develop red colored patches few hours before death; no external growth
Bacteria		
Bacterial disease of digestive organ	*Streptococci* sp./*Staphylococci* sp	Sluggish movement; retarded growth; transparent cephalothoracic region
Septicemia	*Bacillus* sp., *Streptococci* sp., *Staphylococci* sp., *Serratia marcescens*	Sluggish movement; low appetite; swollen thorax; shrinkage; vomiting softened and discolored body
Toxicosis	*Bacillus thuringiensis* var. *Sotto*	Sluggish movement; retarded growth; caesation of feeding; vomiting; paralysis and death; corpse stretch and cephalothoracic region bend like hook.
Microsporidia		
Microsporidiosis/ Pebrine	*N. bombycis*	Flaccid larvae; retarded growth; white pustules all along the length of the silk gland

5.0.7 Cross infectivity of microsporidia isolated from collected insects to the silkworm *B. mori* L.

Microsporidia are prevalent in insects in natural field condition. Insects in nearly all the taxonomic orders are susceptible to microsporidia, but over half of the susceptible insect hosts occur in two orders, Lepidoptera and Diptera (Ghani *et al*., 2013). The chronic infections caused by microsporidia are a significant problem for all types of beneficial insects from honeybees to biological control agents such as parasitoids (Becnel and Andreadis, 2014).

Most of the entomopathogenic microsporidia occur in the genus *Nosema*, and more than 150 described species are found in 12 orders of insects (Becnel and Andreadis, 1999). Microsporidia have been reported from different agricultural and mulberry pests such as *Spodoptera litura, Spodoptera exigua, Helicoverpa armigera, Plutella xylostella, Pieris rapae, Spilosoma oblique* and *Phyllobrotica armata* Baly (Tanada, 1955; Tsai *et al.*, 2003). Reports suggest that *B. mori* is not only infected by the *Nosema* sp. but atleast by 10 other genera *viz.*, *Vairimorpha, Pleistophora, Endoreticulatus, Cystosporogenes, Orthosomella, Thelohania, Octosporea* and *Gurleya* (Zhu *et al.*, 2010; Abe and Fujiwara, 1979).

There are a number of butterflies commonly occurring in the environment which contain microsporidia that are cross-infective to silkworm (Samson *et al.*, 1999a, b). The butterflies belonging to *Catopsilia* genus are frequent visitors to the fields around mulberry gardens and carry microsporidian spores which are found to be infective to silkworm *B. mori*. Natural infection with *N. bombycis* has been reported in several lepidopteran insects such as *Pieris rapae* (Pieridae), *Spodoptera deparvata* (Noctuidae), *Spodoptera exigua* (Noctuidae), *Spodoptera litura* (Noctuidae) and *Trichoplusia ni* (Noctuidae). The mulberry pyralid, *Diaphania pulverulentalis* (Pyralidae) is also known to harbour *N. bombycis* (Sharma *et al.*, 2003). Therefore, keeping the above in view, it is important to test the cross-infectivity of microsporidia isolated from various mulberry and other agricultural insect pests to the mulberry silkworm, *B. mori* L.

Table 5.5: Characteristic features of microsporidians infecting silkworm, *B.mori*, L.

Microsporidian isolates	**Spore shape**	**Spore size (µm)**		**Virulence**
		Length	**Width**	
Nosema bombycis	Oval	3.8	2.6	High
Nosema sp. (NIS-M11)	Oval	3.9	1.9	Low
Nosema sp. (NIS-M14)	Oval	4.1	2.3	High
Nosema sp. (NIK-2r)	Ovidal	3.6	2.8	High
Nosema sp. (NIK-3h)	Ovo-cylindrical	3.8	1.8	Low
Vairimorpha sp. (NIS-M12)	Ovo-cylindrical	4.5	2.0	Low
Vairimorpha sp. (NIK-4m)	Ovo-cylindrical	5.0	2.1	High
Microsporidium sp. (NIS-25)	Oval	4.9	2.8	Low
Pleistophora sp.(NIS-M24)	Oval	2.7	1.6	low
Pleistophora sp.(NIS-M27)	Oval	5.4	3.0	Low
Thelohania sp.(NIS-M32)	Oval	3.4	1.7	Low

(Source: Nataraju, *et. al.,* 2005)

5.1 MATERIALS AND METHODS

5.1.1 Selection of silkworm breed, *B. mori* L.

The popular bivoltine breed of silkworm, CSR2×CSR4 was selected to the study the infectivity of microsporidia isolated from insect pests of mulberry and other agricultural crops. The third instar larvae of silkworm *B. mori* L. were obtained from the Rearing House of the Department of Applied Animal Science, Babasaheb Bhimrao Ambedkar University, Lucknow.

5.1.2 Preparation of different concentrations of isolated microsporidia

The spore concentrations of the stock solutions of each of the isolated microsporidian spores *viz.* M-Dch, M-Cfl, M-Mph, M-Ame, M-Scy and *N. bombycis* were estimated by following standard haemocytometer count method (Cantwell, 1970). Three different concentrations (1×10^5, 1×10^6, 1×10^7 spores/ml) of each of the isolated microsporidian spore was prepared from the quantified stock inoculum by serial dilution for tests.

5.1.3 Infectivity of microsporidian spores to silkworm larva

The infectivity potential of the microsporidian spores isolated from the selected insects (test) and of the *N. bombycis* (control) was tested against the silkworm larva by following the method given by Sharma *et al.* (2003). Each inoculum dose i.e. 1×10^5, 1×10^6 and 1×10^7 spores/ml of M-Dch, M-Cfl, M-Mph, M-Ame, M-Scy and of *N. bombycis* spore solutions formed a treatment regimen. The mulberry leaves (AR-12 variety) were smeared with the respective spore suspension and fed to silkworms on day one of III instar (After II moult) larvae. Another set of larvae inoculated with different concentrations of *N. bombycis* spores was maintained separately for comparison. Each of the inoculum concentrations formed a treatment and for each treatment, two replications of 50 larvae each were maintained. A control batch of silkworm larvae fed on mulberry leaves without any inoculation was also maintained for comparison purposes.

5.1.4 Determination of Median Lethal Concentration (LC_{50})

Silkworm larval mortality rate due to the concerned microsporidia was observed in all the doses after every 24 h upto the spinning i.e. for 16 days post inoculation (16 DPI) and pebrine infection was also checked in the dead larvae under a phase contrast microscope. LC_{50} values were calculated by probit analysis (Finney, 1971). The mortality rate was determined by the following formula:

$$\text{Mortality \%} = \frac{\text{Number of dead larvae}}{\text{Total number of larvae}} \times 100$$

The larval length, larval width and larval weight of the silkworm larva inoculated with M-Dch, M-Cfl, M-Mph, M-Ame and M-Scy spores at a dose concentration of 1×10^7 spores/ml was recorded daily and compared with the batch inoculated with *N. bombycis* spores of the same concentration and another with control batch (reared without inoculation of any microsporidian pathogen). Further, in the silkworm batches inoculated with spore concentration of 1×10^5 spores/ml and 1×10^6 spores/ml of M-Dch, M-Cfl, M-Mph, M-Ame and M-Scy and *N. bombycis*, the larval length, larval width and larval weight were measured one day prior to spinning and compared to that of healthy batches of silkworm larva. The data was statistically analyzed by one way ANOVA using IBM SPSS 20 Software.

5.1.5 Effect of microsporidian infection on pupa and cocoon characters

In the silkworm batches inoculated with M-Dch, M-Cfl, M-Mph, M-Ame and M-Scy spores at a dose concentration of 1 $\times 10^7$ spores/ml, the weight of the cocoon and that of the shell were recorded and the shell ratio % was calculated by the formula given below.

$$\text{Shell ratio } \% = \frac{\text{Shell weight (gm)}}{\text{Cocoon weight (gm)}} \times 100$$

The filament length of the cocoon was also recorded. The results of the test microsporidia were compared with the batch inoculated with *N. bombycis* (standard) of the same dose concentration (1 $\times 10^7$ spores/ml) and another with control batch (reared without inoculation of any microsporidian pathogen). The data was statistically analyzed by one way ANOVA using IBM SPSS 20 Software.

5.2 RESULTS

5.2.1 Cross-infectivity and LC_{50} value of the isolated microsporidia against *B. mori*

The infectivity of the microsporidian isolates M-Dch, M-Cfl, M-Mph, M-Ame, M-Scy and *N. bombycis* (Nbo) were checked against the mulberry silkworm *B. mori* (CSR2×CSR4). The results showed that the there was no larval mortality due to microsporidiosis in the silkworm batches inoculated with M-Ame and M-Scy spores while mortality was recorded in the silkworm batches inoculated with M-Dch, M-Cfl, M-Mph and *N. bombycis* (Nbo) and the dead larva were found to be positive for the infection. The percentages of mean larval mortality were determined at 16 days post inoculation for each of the respective microsporidian inoculated batches at the three different concentrations *viz*. 1×10^5 spores/ml, 1×10^6 spores/ml and 1×10^7 spores/ml. On the basis of results, in M-Dch inoculated batches of silkworm larva, the mortality rates of 11%, 19% and 35% were recorded at doses of 1×10^5 spores/ml, 1×10^6 spores/ml and 1×10^7 spores/ml respectively. Similarly in M-Cfl and M-Mph inoculated batches of silkworm, larval mortality percentages of 12% and 9% were recorded respectively at a dose of 1×10^5 spores/ml, where as larval mortality of 25%

and 16% were recorded respectively at a dose of 1×10^6 spores/ml and in both the batches maximum larval mortality rate was recorded as 40% and 31% at the higher dose concentration of 1×10^7 spores/ml. Further, in silkworm batches inoculated with standard strain of *N. bombycis*, larval mortality rates of 21%, 43% and 81% were recorded at dose concentrations of 1×10^5 spores/ml, 1×10^6 spores/ml and 1×10^7 spores/ml respectively (Table 5.6). At 16 days post inoculation, the LC_{50} value of M-Dch, M-Cfl and M-Mph spores against larval mortality of *B. mori* were calculated as 3.2×10^7 spores/ml, 2.69×10^8 spores/ml and 9.67×10^7 spores/ml respectively whereas the LC_{50} value of *N. bombycis* (Nbo) against larval mortality of *B. mori* was calculated as 1.1×10^6 spores/ml (Table 5.6, Figure 5.2, 5.3, 5.4 and 5.5). The results indicated that among the isolated microsporidia, M-Cfl spores were more virulent to silkworm *B. mori* followed by M-Dch and M-Mph spores but less virulent when compared with *N. bombycis* as highest larval mortality (81%) was recorded in silkworm batches inoculated with *N. bombycis* spores.

Table 5.6: LC50 value of the infection of isolated microsporidia and *N. bombycis* against the silkworm, *B. mori* L.

Treatment Batches	**Conc. of Microsporidia (spores/ml)**	**Log Conc.**	**Larval mortality due to microsporidian infection (%)**	**Probit Value**	**LC_{50}value (spores/ml)**
M-Dch	1×10^5	5	11	3.77	3.2×10^7
	1×10^6	6	19	4.12	
	1×10^7	7	35	4.61	
M-Cfl	1×10^5	5	12	3.82	2.69×10^7
	1×10^6	6	25	4.33	
	1×10^7	7	40	4.75	
M-Mph	1×10^5	5	9	3.66	9.67×10^7
	1×10^6	6	16	4.01	
	1×10^7	7	31	4.50	
Nosema bombycis	1×10^5	5	21	4.19	1.1×10^6
	1×10^6	6	43	4.82	
	1×10^7	7	81	5.88	

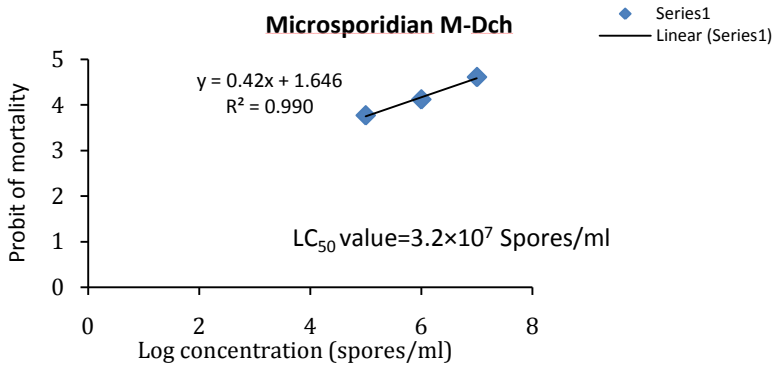


Figure 5.2: Probit graphs for larval mortality due to M-Dch microsporidia on *B. mori* L., 16th day post inoculation

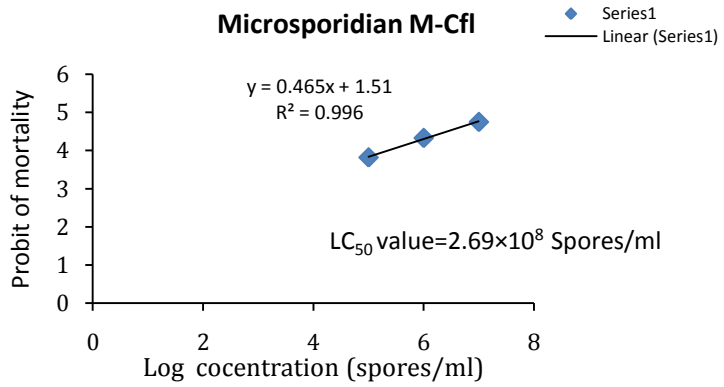


Figure 5.3: Probit graphs for larval mortality due to M-Cfl microsporidia on *B. mori* L., 16th day post inoculation

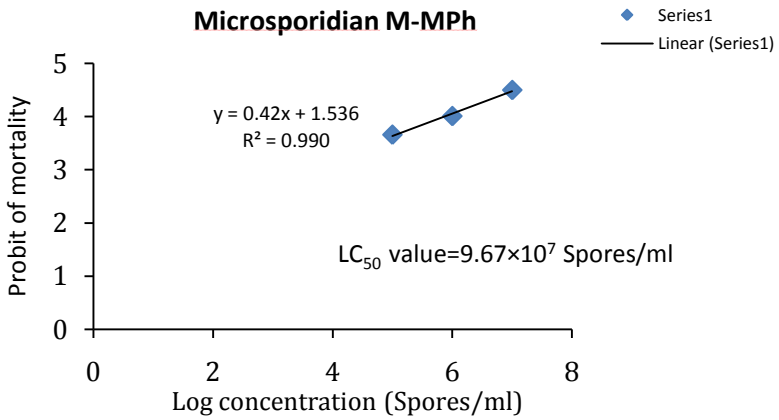


Figure 5.4: Probit graphs for larval mortality due to M-Mph microsporidia on *B. mori* L., 16th day post inoculation

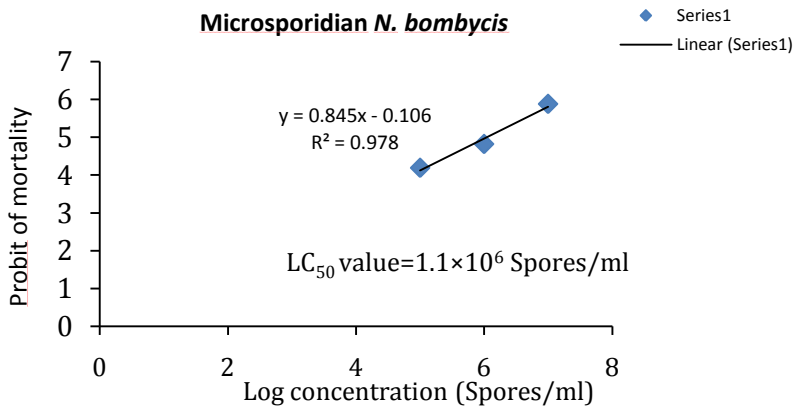


Figure 5.5: Probit graphs for larval mortality due to *N. bombycis* on *B. mori* L., 16th day Post inoculation

5.2.2 Impact of infection of the isolated microsporidia on the morphology of the silkworm, *B. mori* L.

5.2.2.1 Larval length: The results with regard to the impact of the infection caused by different isolated microsporidia on the daily increase in larval length of silkworm (CSR2×CSR4 breed) are presented in Table 5.7. The data revealed that there was no significant reduction in larval length of silkworm in the batches inoculated with microsporidia M-Ame and M-Scy and that these microsporidia were not pathogenic to the silkworm *B. mori* L. The observations showed that there was no significant impact of the infection by the isolated microsporidia and *N. bombycis* on the progressive increase in the larval lengths upto 7th day post inoculation. However, from the 8th day post inoculation (PI) onwards, significant progressive reduction in larval lengths was recorded in the batches inoculated with M-Cfl (3.46 cm) and *N. bombycis* (33.32 cm) in comparison to the healthy control batches wherein the larval length of 3.62 cm was observed on 8th day PI. In the batches inoculated with the microsporidia M-Dch, M-Mph, M-Ame, M-Scy, there was not observed any significant reduction in the larval length on 8th day post inoculation which were recorded as 3.52 cm, 3.56 cm, 3.62 cm and 3.60 cm respectively. Further, on the 9th and 10th day PI, significant reduction in the larval length was observed in the batches inoculated with microsporidia M-Dch, M-Cfl and *N. bombycis* respectively as compared to the healthy control batches. On the 9th day PI, the larval length was recorded as 3.56 cm, 3.52 cm and 3.44 cm in the batches inoculated with M-Dch, M-Cfl and *N. bombycis* respectively as compared to

the 3.7 cm larval length recorded in healthy control batch. On the 10th day PI, the larval length was recorded as 3.66 cm, 3.64 cm and 3.62 cm in the batches inoculated with M-Dch, M-Cfl and *N. bombycis* respectively compared to the 3.78 cm larval length in the healthy control batch. From 11th to 16th day PI significant reduction was observed in the larval length in the batches of the silkworm larva inoculated with microsporidia M-Dch, M-Cfl, M-Mph and *N. bombycis* respectively and the larval length ranged from 3.62 cm to 6.40 cm when compared to healthy control batches where the value ranged from 3.86 cm to 6.60 cm. The batches of larva inoculated with microsporidium M-Ame and M- Scy showed no pathogenic effect and the increase in larval lengths in these batches indicated that there were no significant changes in the length value when compared to healthy control batches of silkworm larvae. The impact of the microsporidian infections on the larval lengths was more pronounced with the progression of infection and on the 15th day of post inoculation, the lowest larval length was recorded in the batches inoculated with M-Cfl (5.70 cm) followed by M-Dch (5.84 cm) and M-Mph (6.00 cm) whereas the same was recorded as 5.48 cm in *N. bombycis* inoculated batches and the healthy control batches (6.14 cm). One day prior to the spinning, *i.e.* on the 16th day PI, the larval lengths were recorded as 6.26 cm, 6.02 cm, 6.40 cm, 6.56 cm, 6.58 cm and 5.54 cm in the batches inoculated with M-Dch, M-Cfl, M-Mph, M-Ame, M-Scy and *N. bombycis* respectively as compared to the healthy control batches wherein a larval length of 6.60 cm was recorded.

The decrease in the larval length of the batches inoculated with the microsporidia *viz*. M-Dch, M-Cfl, M-Mph and *N. bombycis* compared to that of the healthy control batches from the 1st day of post inoculation till the 16th day of post inoculation i.e. one day prior to the onset of spinning is graphically represented in Figure 5.6.

5.2.2.2 Larval width: The impact of infection of the isolated microsporidia on the daily increase in larval width of the silkworm is presented in Table 5.8. The data showed no significant impact of infection by the isolated microsporidia on the progressive increase in larval width up to the 9th day post inoculation. However, in the batches inoculated with *N. bombycis*, the increase in larval widths were significantly reduced from the 8th day PI onwards and were recorded as 0.56 cm, 0.60 cm, 0.62 cm,

0.62 cm, 0.64 cm and 0.74 cm against the larval widths of 0.66 cm, 0.70 cm, 0.74 cm, 0.74 cm, 0.76 cm and 0.84 cm in the healthy control batches on 8th, 9th, 10th, 11th, 12th and 13th day PI respectively. In case of M-Cfl inoculated batches, significant reduction in larval widths was observed from 10th day to the 16th day post inoculation and the values were recorded as 0.64 cm, 0.68 cm, 0.68 cm, 0.76 cm, 0.84 cm, 0.9 cm and 0.96 cm respectively. From 14th day to 15th day post inoculation, significant reduction of larval widths was observed in both M-Dch and M-Mph inoculated batches of silkworm larva. In both M-Dch and M-MPh batches, the value of larval width were recorded as 0.88 cm, 0.96 cm on 14th and 15th day post inoculation, whereas on 16th day of PI, the larval width was recorded as 1.02 in case of M-Dch inoculated batches and 1.04 cm in case of M-MPh inoculated batches. In contrast, in the healthy control batches of silkworm larva, the larval width was recorded as 1.00 cm, 1.12 cm and 1.20 cm respectively on 14th, 15th and 16th day post inoculation. As compared to the healthy batches, a significant reduction of larval width was observed in the batches inoculated with standard strain of *N. bombycis* wherein the values were recorded as 0.76 cm, 0.82 cm and 0.84 cm respectively on 14th, 15th and 16th day post inoculation.

The decrease in the larval width in the batches inoculated with the microsporidia *viz*. M-Dch, M-Cfl, M-Mph and *N. bombycis* as compared to that in the healthy control batches from first day post inoculation till 16th day post inoculation i.e. one day prior to the onset of spinning is graphically represented in Figure 5.7.

5.2.2.3 Larval weight: The impact of infection of the isolated microsporidia on daily increase in larval weight is presented in Table 5.9. The larval weights of 5 larvae were measured as a group from each of the inoculated batches infected by the respective isolated microsporidia. The data revealed that there was no significant reduction in larval weight of silkworm batches inoculated with microsporidia M-Ame and M-Scy and it was also observed that these microsporidia were non infective to the silkworm *B. mori* L. Further, no significant effects of the infection of the other isolated microsporidia *viz*. M-Dch, M-Cfl and M-Mph, was observed on the progressive increase in larval weight from the day of inoculation up to 10th day of PI . On the other hand, in case of *N. bombycis* inoculated batches, although no significant impact of infection on larval weight upto 7th day of PI, from 8th day PI onwards, it was

observed that the progressive increase in larval weight was slightly reduced and it was recorded as 3.74 g, 4.33 g, 4.50, 4.61 and 4.80 g on 8th, 9th, 10$^{th.}$, 11th, and 12th day PI respectively. In the M-Cfl inoculated batches of silkworm, larval weight was significantly reduced from 11th day PI onwards whereas in both the M-Dch and M-Mph inoculated batches, the larval weights were significantly reduced from 13th day of PI onwards in comparison to the larval weights of the healthy control batches. On 13th day PI, larval weight of 11.06, 10.04 and 11.18 g was recorded in the batches inoculated with M-Dch, M-Cfl and M-Mph respectively whereas the same in case of *N. bombycis* inoculated batches was recorded as 9.24 g as against the larval weight of 11.67 g of healthy control batches, thus showing a significant reduction in the progressive increase in larval weights. Further on the 14th and 15th day of PI the larval weight were recorded as 14.91 g and 16.05 g in M-Dch inoculated batches, 14.24 g and 15.15 g in M-Cfl inoculated batches, and 15.00 g and 16.72 g in M-Mph inoculated batches of silkworm respectively. This reduction in larval weight was comparatively more pronounced in *N. bombycis* inoculated batches, where larval weights of 12.58 and 13.53 g on 13th and 14th day PI respectively were recorded. In the healthy control batches, the larval weights of 15.06 g and 17.67 g respectively were recorded, which was significantly higher than that observed in the inoculated batches. On one day prior to the onset of spinning (i.e. 16th day PI), the adverse impact of microsporidian infection on larval weight was further-more pronounced. The lowest larval weight was recorded in the batches inoculated with M-Cfl (17.73 g) followed by M-Dch (18.03 g) and M-Mph (18.16 g) inoculated batches. In case of the batches inoculated with *N. bombycis*, 13.97 g larval weight was recorded as compared to the larval weight of 19.25 g in the healthy control batches. The percentage reduction in the larval weight of the inoculated batches compared to that of the healthy control batches on one day prior to the onset of spinning is graphically represented in Figure 5.8.

5.2.3 Effect of microsporidian infection on the economic characters of silkworm *B. mori* L. (CSR2×CSR4 breed)

The impact of infections of M-Dch, M-Cfl and M-Mph, M-Ame, M-Scy and *N. bombycis* spores on economic characters of silkworm *B. mori* L. at different inoculation doses is furnished in Table 5.10, 5.11 and Table 5.12 respectively. It was

observed that the microsporidian isolates *viz.* M-Dch, M-Cfl, M-Mph and *N. bombycis* caused significant effects on the economic characters of silkworm where as the isolates M-Ame and M-Scy did not cause any infection in the silkworm larva and therefore did not cause any significant effect on the economic characters of silkworm as compared to the economic characters of healthy control batches of silkworm larva. A comparison with respective healthy control batch indicated that the infections due to the three microsporidia isolates *viz.* M-Dch, M-Cfl and M-Mph adversely impacted prominent cocoon characteristics and resulted in lowered larval weight, cocoon weight, shell weight and filament length.

5.2.3.1 Larval weight: The weight of group of 5 mature larvae was significantly affected by the micosporidian isolates (M-Dch, M-Cfl and M-Mph) in all the three doses i.e. at 1×10^5 spores/ml, 1×10^6 spores/ml and 1×10^7 spores/ml. The weights of groups of 5 mature larvae were recorded as 18.62 g, 18.36 g and 18.69 g respectively for the M-Dch, M-Cfl and M-Mph inoculated batches with a dose concentration of 1×10^5 spores/ml where as the weight of 5 mature larvae in healthy control batches was recorded as 19.25 g. However, the reduction in 5 mature larval weight was more pronounced in the silkworm batches inoculates with *N. bombycis* spores at the dose of 1×10^5 spores/ml, wherein the value was calculated as 15.02 g. The microsporidian infection was not detected in the batches inoculated with M-Ame and M-Scy inoculated silkworm batches where the weight of 5 mature larva were recorded as 19.25 g and 19.25 g respectively and the same value was also recorded in the healthy control batches (Table 5.10, Figure 5.9).

Furthermore, at the medium dose concentration of 1×10^6 spores/ml, a significant reduction in the weights of 5 mature larvae in the batches inoculated with M-Dch, M-Cfl, M-Mph and *N. bombycis* spores was observed where the values were measured as 18.36 g, 18.11 g, 18.50 g and 14.13 g respectively. In comparison, the weights of 5 mature larvae of healthy control batches was recorded as 19.25 g (Table 5.11, Figure 5.10). The isolated microsporidia caused significant impact on the weight of 5 mature larvae of the batches inoculated with M-Dch, M-Cfl, M-Mph and *N. bombycis* spores at the highest dose concentration of 1×10^7 spores/ml and the values were recorded as 18.03 g, 17.73 g, 18.16 g and 13.97 g respectively as compared to

weight of 5 mature larvae recorded as 19.25 g in the healthy control batches of silkworm larva (Table 5.12, Figure 5.11).

5.2.3.2 Cocoon Weight: The weights of the cocoons was not affected in the silkworm batches inoculated with M-Dch, M-Mph spores of dose concentration 1×10^5 spores/ml where as the weights of cocoons were significantly reduced in the batches inoculated with M-Cfl spore at the same dose concentration i.e. 1×10^5 spores/ml (1.57 g) when compared to the weight of healthy cocoons (1.59 g) from control batch (Table 5.10, Figure 5.12). However, the cocoon weight was significantly reduced in silkworm batches inoculated with isolated microsporidia (M-Dch, M-Cfl and M-Mph) at a dose concentration of 1×10^6 spores/ml wherein the cocoon weights were measured as 1.57 g, 1.56 g and 1.57 g respectively whereas the weight of healthy cocoons was recorded as 1.59 g (Table 5.11, Figure 5.13). Furthermore, in the silkworm batches inoculated with isolated microsporidia of doses 1×10^7 spores/ml, it was found that the cocoon weight was significantly affected in the M-Dch, M-Cfl and M-Mph inoculated batches (Table 5.12, Figure 5.14). Among these, the reduction in cocoon weight was more pronounced in M-Cfl (1.50 g) inoculated batches followed by both M-Dch (1.52 g) and M-Mph (1.52 g) inoculated batches when compared to the cocoon weight (1.59 g) of healthy control batches. Also, the effects on the cocoon weight was more pronounced in the silkworm batches inoculates with the standard *N. bombycis* where in the cocoon weight were measured respectively as 1.53 g, 1.50 g and 1.41 g in the batches inoculated with doses 1×10^5 spores/ml, 1×10^6 spores/ml and 1×10^7 spores/ml when compared to the weight of healthy cocoons from control batch where the value was measured as 1.59 g.

5.2.3.3 Shell Weight: The shell weight was not affected in the silkworm batch inoculated with M-Mph spores at dose concentration of 1×10^5 spores/ml where as the shell weight was significantly reduced in the batches inoculated with M-Dch and M-Cfl spore at the same doses concentration where the shell weight measured respectively as 0.33 g and 0.32 g as compared to the shell weight (0.35 g) of healthy control batch (Table 5.10, Figure 5.15). However, the shell weight reduced significantly in the silkworm batches inoculated with isolated microsporidia *viz.* M-Dch, M-Cfl and M-Mph at a dose concentration of 1×10^6 spores/ml wherein the shell weight was measured as 0.32 g, 0.31 g and 0.33 g respectively whereas the shell

weight of healthy cocoon was recorded as 0.35 g (Table 5.11, Figure 5.16). In the silkworm batches inoculated with isolated microsporidia of doses 1×10^7 spores/ml, it was found that the shell weight was significantly affected in the M-Dch, M-Cfl and M-Mph inoculated batches where the shell weight was more affected in M-Cfl (0.29 g) inoculated batches followed by both M-Dch (0.30 g) and M-Mph (0.30 g) inoculated batches when compared to the shell weight (0.35 g) of healthy control batches (Table 5.12, Figure 5.17). Again, the decrease in the shell weight was more pronounced in the silkworm batches inoculates with the standard *N. bombycis* strain where in the shell weights were measured as 0.30 g, 0.28 g and 0.26 g in the batches inoculated with doses of 1×10^5 spores/ml, 1×10^6 spores/ml and 1×10^7 spores/ml respectively when compared to the shell weight of healthy cocoons from control batch where the value was measured as 0.35 g.

5.2.3.4 Shell Ratio Percentage (SR%): The infection due to the isolated microsporidia (M-Dch, M-Cfl and M-Mph) led to the reduction in the shell ratio percentage of the treated batches when compared to the healthy control batch.

The shell ratio percentage was not affected in the silkworm batch inoculated with M-Dch and M-Mph spores at dose concentration of 1×10^5 spores/ml where as the shell ratio percentage was significantly reduced in the batches inoculated with M-Cfl spores at the same dose concentration where the SR % was measured as 20.3% as compared to the shell ratio percentage of 22.17% recorded in the healthy control batch (Table 5.10, Figure 5.18). However, the shell ratio percentage was significantly reduced in the silkworm batches inoculated with isolated microsporidia *viz.* M-Dch, M-Cfl and M-Mph at a dose concentration of 1×10^6 spores/ml wherein the shell ratio percentages were measured as 20.53%, 19.6% and 20.73% respectively. The shell ratio percentage of healthy cocoon was recorded as 22.17% (Table 5.11, Figure 5.19). Furthermore, in the silkworm batches inoculated with isolated microsporidia of doses 1×10^7 spores/ml, it was found that the shell ratio percentages were significantly affected in the M-Dch, M-Cfl and M-Mph inoculated batches where the SR % was more affected in M-Cfl (19.15%) inoculated batch followed by M-Dch (19.73%) and M-Mph (19.96%) inoculated batches when compared to the shell ratio percentage (22.17%) of healthy control batches (Table 5.12, Figure 5.20). Again, the effect on the shell ratio percentage was much more pronounced in the silkworm batches inoculated

with the standard *N. bombycis* strain where in the SR % were measured as 18.91%, 18.62% and 18.20% in the batches inoculated with doses of 1×10^5 spores/ml, 1×10^6 spores/ml and 1×10^7 spores/ml respectively when compared to the shell ratio percentage of healthy cocoon from control batch where the value was measured as 22.17%.

5.2.3.5 Filament Length: The infection due to the isolated microsporidia (M-Dch, M-Cfl and M-Mph) led to the significant reduction in the filament length of the treated batches when compared to the healthy control batch. The filament length was more affected in the silkworm batch inoculated with M-Cfl batches followed by M-Dch and M-Mph inoculated batches in all the three doses. Again, in the silkworm batches inoculated with the isolated microsporidia *viz.* M-Dch, M-Cfl and M-Mph of dose concentration 1×10^5 spores/ml, the filament lengths were measured as 843 m, 889 m and 902 m respectively where as the filament length of healthy cocoon from control batches was measured as 966 m (Table 5.10, Figure 5.21). The filament length was significantly more reduced in the silkworm batches inoculated with higher doses of the microsporidia. In the silkworm batches inoculated with M-Dch, M-Cfl and M-Mph spores at dose concentration of 1×10^6 spores/ml, the filament lengths were measured as 859 m, 807 m and 868 m respectively (Table 5.11, Figure 5.22). Further, the value of the filament lengths was reduced to 841 m, 790 m and 843 m respectively in the M-Dch, M-Cfl and M-Mph inoculated batches of silkworm at dose concentration of 1×10^7 spores/ml (Table 5.12, Figure 5.23). Again, the effect of microsporidia on the filament length was more pronounced in the silkworm batches inoculated with the standard strain of *N. bombycis* strain wherein the filament lengths were measured as 633 m, 626 m and 608 m for the batches inoculated with the dose concentrations of 1×10^5 spores/ml, 1×10^6 spores/ml and 1×10^7 spores/ml respectively where as the filament length of healthy cocoons from control batch was measured as 966 m.

Table 5.7: Effect of isolated microsporidian infection on larval length of the silkworm, *Bombyx mori* L. (CSR2×CSR4 breed)

Microsporidian Isolates	**Larval length (cm) Days post Inoculation (1× 10^7 Spores/ml inoculated batches)**															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
M-Dch	1.34 ± 0.05	1.80 ± 0.07	2.14 ± 0.11	2.24 ± 0.08	2.30 ± 0.08	2.38 ± 0.07	3.16 ± 0.05	3.52 ± 0.04	3.56* ± 0.05	3.66* ± 0.05	3.70* ± 0.07	3.70* ± 0.07	4.80* ± 0.07	5.24* ± 0.08	5.84* ± 0.05	6.26* ± 0.08
M-Cfl	1.34 ± 0.05	1.80 ± 0.07	2.12 ± 0.05	2.24 ± 0.05	2.28 ± 0.11	2.36 ± 0.05	3.14 ± 0.05	3.46* ± 0.05	3.52* ± 0.04	3.64* ± 0.05	3.66* ± 0.08	3.70* ± 0.07	4.66* ± 0.05	5.10* ± 0.07	5.70* ± 0.07	6.02* ± 0.04
M-Mph	1.34 ± 0.05	1.82 ± 0.04	2.14 ± 0.05	2.24 ± 0.05	2.3 ± 0.07	2.38 ± 0.07	3.16 ± 0.05	3.56 ± 0.05	3.64 ± 0.05	3.70 ± 0.07	3.70* ± 0.07	3.74* ± 0.05	5.06* ± 0.05	5.32* ± 0.05	6.00* ± 0.10	6.40* ± 0.07
M-Ame	1.34 ± 0.05	1.82 ± 0.04	2.16 ± 0.05	2.24 ± 0.07	2.30 ± 0.07	2.40 ± 0.08	3.18 ± 0.04	3.60 ± 0.04	3.66 ± 0.08	3.70 ± 0.07	3.82 ± 0.04	3.98 ± 0.08	5.26 ± 0.05	5.62 ± 0.04	6.08 ± 0.08	6.56 ± 0.05
M-Scy	1.34 ± 0.05	1.80 ± 0.07	2.16 ± 0.07	2.24 ± 0.08	2.30 ± 0.08	2.40 ± 0.07	3.18 ± 0.04	3.62 ± 0.07	3.68 ± 0.08	3.78 ± 0.04	3.84 ± 0.05	4.00 ± 0.07	5.28 ± 0.04	5.64 ± 0.05	6.10 ± 0.07	6.58 ± 0.04
Nosema bombycis	1.34 ± 0.05	1.80 ± 0.07	2.12 ± 0.05	2.18 ± 0.04	2.22 ± 0.04	2.30 ± 0.07	3.14 ± 0.05	3.32* ± 0.08	3.44* ± 0.05	3.62* ± 0.04	3.62* ± 0.04	3.64* ± 0.05	4.42* ± 0.08	4.86* ± 0.05	5.48* ± 0.04	5.54* ± 0.05
Healthy Control	1.34 ± 0.05	1.82 ± 0.04	2.16 ± 0.05	2.24 ± 0.05	2.30 ± 0.07	2.40 ± 0.07	3.18 ± 0.04	3.62 ± 0.04	3.7 ± 0.07	3.78 ± 0.04	3.86 ± 0.05	4.00 ± 0.07	5.28 ± 0.04	5.64 ± 0.05	6.14 ± 0.11	6.60 ± 0.07

Data are expressed as Mean ±SD (n=5), *statistically significant (P<0.05) compared to healthy control larva by One way ANOVA using IBM SPSS 20 Software.

Table 5.8: Effect of isolated microsporidian infection on larval width of the silkworm, *Bombyx mori* L. (CSR2×CSR4 breed)

Microsporidian Isolates	**Larval Width (cm) Days post Inoculation (1× 10^7 Spores/ml inoculated batches)**															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	16
M-Dch	0.30 ± 0.00	0.38 ± 0.04	0.40 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.50 ± 0.00	0.62 ± 0.04	0.64 ± 0.05	0.68 ± 0.04	0.70 ± 0.00	0.70 ± 0.00	0.80 ± 0.00	0.88* ± 0.04	0.96* ± 0.05	1.02* ± 0.04
M-Cfl	0.30 ± 0.00	0.38 ± 0.04	0.40 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.50 ± 0.00	0.62 ± 0.04	0.64 ± 0.05	0.64* ± 0.05	0.68* ± 0.04	0.68* ± 0.04	0.76* ± 0.01	0.84* ± 0.05	0.9* ± 0.07	0.96* ± 0.08
M-Mph	0.30 ± 0.00	0.38 ± 0.04	0.40 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.50 ± 0.00	0.62 ± 0.04	0.64 ± 0.05	0.68 ± 0.04	0.70 ± 0.00	0.70 ± 0.00	0.80 ± 0.00	0.88* ± 0.04	0.96* ± 0.05	1.04* ± 0.05
M-Ame	0.30 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.50 ± 0.00	0.64 ± 0.05	0.68 ± 0.04	0.70 ± 0.00	0.70 ± 0.00	0.72 ± 0.04	0.82 ± 0.04	0.96 ± 0.05	1.08 ± 0.05	1.16 ± 0.05
M-Scy	0.30 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.50 ± 0.00	0.64 ± 0.05	0.68 ± 0.04	0.72 ± 0.04	0.72 ± 0.04	0.72 ± 0.04	0.82 ± 0.04	0.98 ± 0.08	1.10 ± 0.04	1.18 ± 0.08
Nosema bombycis	0.30 ± 0.00	0.38 ± 0.04	0.40 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.50 ± 0.00	0.56* ± 0.05	0.60* ± 0.00	0.62* ± 0.04	0.62* ± 0.04	0.64* ± 0.05	0.74* ± 0.05	0.76* ± 0.05	0.82* ± 0.04	0.84* ± 0.05
Healthy Control	0.30 ± 0.00	0.4 ± 0.04	0.40 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.5 ± 0.00	0.66 ± 0.05	0.7 ± 0.00	0.74 ± 0.05	0.74 ± 0.05	0.76 ± 0.05	0.84 ± 0.05	1.00 ± 0.07	1.12 ± 0.04	1.20 ± 0.07

Data are expressed as Mean ±SD (n=5), *statistically significant (P<0.05) compared to healthy control larva by One way ANOVA using IBM SPSS 20 Software.

Table 5.9: Effect of isolated microsporidian infection on larval weight of the silkworm, *Bombyx mori* L. (CSR2×CSR4 breed)

Microsporidian Isolates	**Larval Weight (g/5 larva) Days post Inoculation (1× 10^7 Spores/ml inoculated batches)**															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	16
M-Dch	0.22 ± 0.01	0.55 ± 0.01	0.81 ± 0.01	1.06 ± 0.01	1.08 ± 0.01	1.09 ± 0.01	2.60 ± 0.01	3.84 ± 0.01	4.52 ± 0.01	4.79 ± 0.01	4.99 ± 0.01	5.30 ± 0.01	11.06* ± 0.01	14.91* ± 0.06	16.05* ± 0.01	18.03* ± 0.01
M-Cfl	0.22 ± 0.01	0.53 ± 0.02	0.81 ± 0.01	1.04 ± 0.01	1.07 ± 0.01	1.09 ± 0.01	2.60 ± 0.01	3.84 ± 0.01	4.51 ± 0.02	4.78 ± 0.02	4.98* ± 0.01	5.30* ± 0.01	10.04* ± 0.03	14.24* ± 0.02	15.15* ± 0.03	17.73* ± 0.02
M-Mph	0.22 ± 0.01	0.55 ± 0.01	0.81 ± 0.02	1.05 ± 0.01	1.08 ± 0.01	1.09 ± 0.01	2.60 ± 0.01	3.84 ± 0.01	4.52 ± 0.01	4.79 ± 0.01	5.00 ± 0.01	5.31 ± 0.01	11.18* ± 0.04	15.00* ± 0.04	16.72* ± 0.06	18.16* ± 0.02
M-Ame	0.22 ± 0.01	0.54 ± 0.01	0.81 ± 0.01	1.05 ± 0.01	1.07 ± 0.01	1.09 ± 0.01	2.61 ± 0.01	3.85 ± 0.01	4.53 ± 0.01	4.80 ± 0.01	5.00 ± 0.01	5.32 ± 0.01	11.66 ± 0.01	15.05 ± 0.01	17.63 ± 0.04	19.24 ± 0.01
M-Scy	0.22 ± 0.01	0.54 ± 0.01	0.81 ± 0.01	1.05 ± 0.01	1.07 ± 0.01	1.09 ± 0.01	2.61 ± 0.01	3.85 ± 0.01	4.53 ± 0.01	4.80 ± 0.01	5.00 ± 0.01	5.33 ± 0.01	11.66 ± 0.02	15.05 ± 0.01	17.64 ± 0.01	19.24 ± 0.02
Nosema bombycis	0.22 ± 0.01	0.53 ± 0.02	0.81 ± 0.01	1.04 ± 0.01	1.07 ± 0.01	1.09 ± 0.01	2.60 ± 0.01	3.74* ± 0.02	4.33* ± 0.01	4.50* ± 0.02	4.61* ± 0.02	4.80* ± 0.01	9.24* ± 0.01	12.58* ± 0.01	13.53* ± 0.02	13.97* ± 0.02
Healthy Control	0.22 ± 0.01	0.54 ± 0.01	0.82 ± 0.01	1.05 ± 0.01	1.07 ± 0.02	1.10 ± 0.01	2.61 ± 0.01	3.85 ± 0.01	4.53 ± 0.02	4.80 ± 0.01	5.02 ± 0.01	5.33 ± 0.01	11.67 ± 0.02	15.06 ± 0.01	17.67 ± 0.02	19.25 ± 0.01

Data are expressed as Mean ±SD (n=5), *statistically significant (P<0.05) compared to healthy control larva by One way ANOVA using IBM SPSS 20 Software.

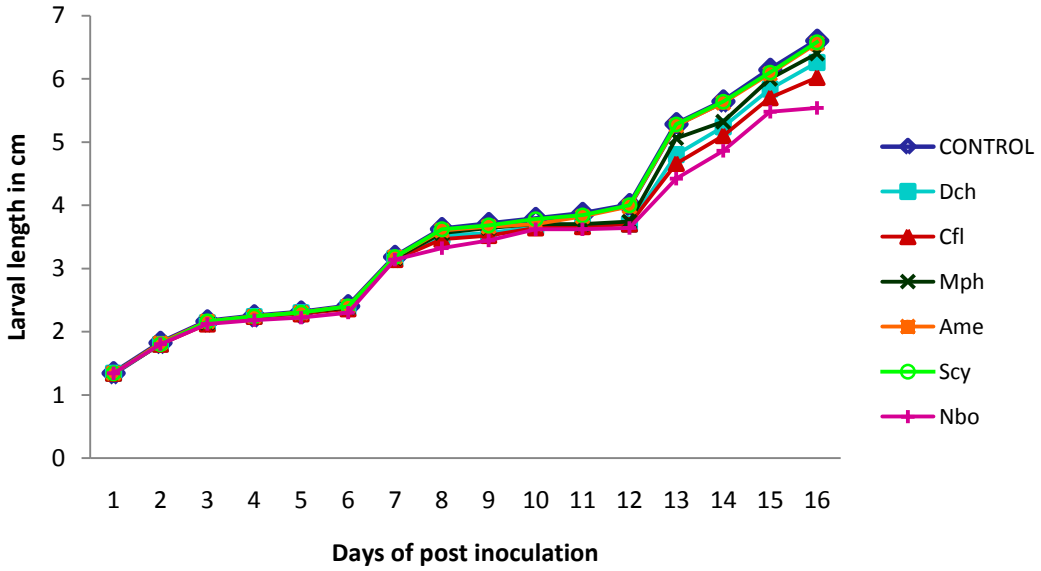


Figure 5.6: Reduction in larval length of silkworm in microsporidian inoculated batches compared to that of the healthy control (One day prior to the onset of spinning)

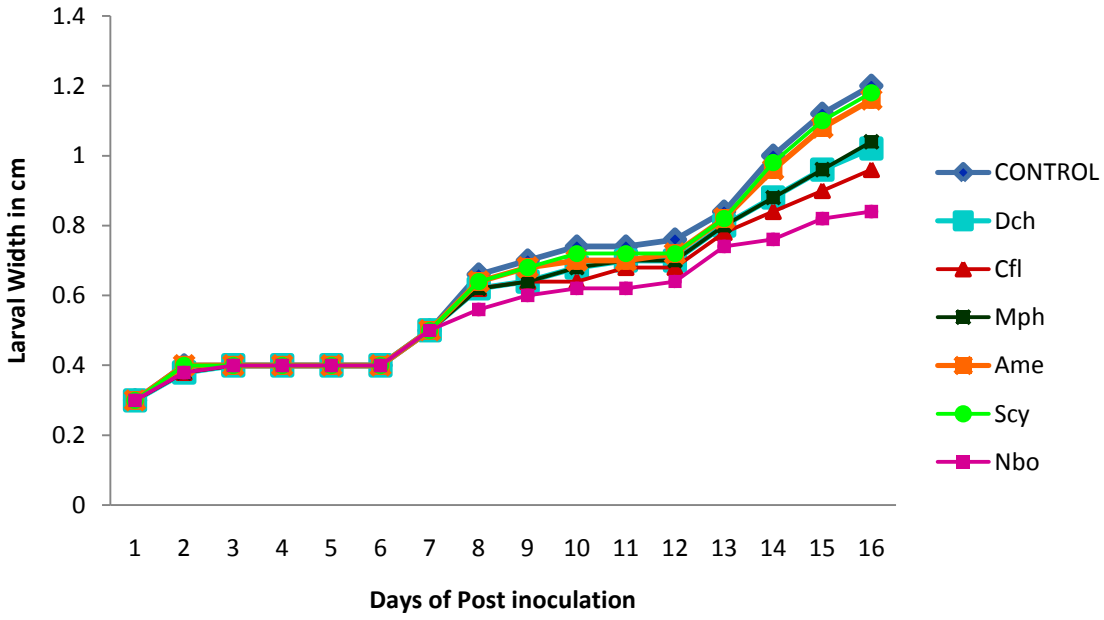


Figure 5.7: Reduction in larval width of silkworm in microsporidian inoculated batches compared to that of the healthy control (One day prior to the onset of spinning)

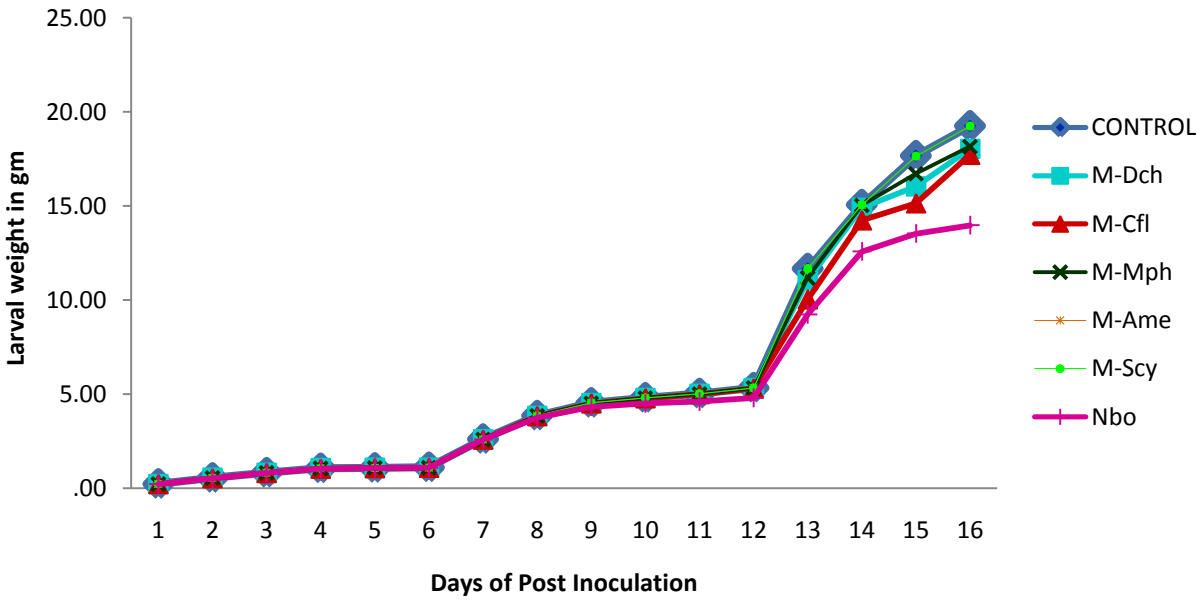


Figure: 5.8: Reduction in larval weight of silkworm in microsporidian inoculated batches compared to that of the healthy control (One day prior to the onset of spinning)

Table 5.10: Effect of Microsporidian isolates (1× 10^5 spores/ml) on larval and cocoon characters of the silkworm, *B. mori L.*

Microsporidian isolates (1× 10^5 spores/ml)	**5 Mature larval weight (g)**	**Cocoon Weight (g)**	**Shell Weight (g)**	**Shell Ratio (SR) %**	**Filament length (m)**
M-Dch	18.62±0.02*	1.58±0.01	0.33±0.01*	21.16±0.46	889±2.65*
M-Cfl	18.36±0.02*	1.57±0.01*	0.32±0.01*	20.3±0.7*	843±1.53*
M-Mph	18.69±0.01*	1.58±0.01	0.34±0.01	21.35±0.41	902±1.53*
M-Ame	19.25±0.01	1.59±0.01	0.35±0.01	22.01±0.70	953±1.52
M-Scy	19.25±0.01	1.59±0.01	0.35±0.01	22.33±0.31	953±1.53
N. bombycis	15.02±0.03*	1.53±0.01*	0.30±0.01*	18.91±0.60*	633±3.36*
Healthy Control	19.25±0.01	1.59±0.01	0.35±0.01	22.17±0.70	966±1.00

Data are expressed as Mean ±SD (n=5), *statistically significant (P<0.05) compared to healthy control larva by One way ANOVA using IBM SPSS20 Software.

Table 5.11: Effect of Microsporidian isolates (1×10^6 spores/ml) on larval and cocoon characters of the silkworm, *B. mori* L.

Microsporidian isolates (1×10^6 spores/ml)	**5 Mature larval weight**	**Cocoon Weight (g)**	**Shell Weight (g)**	**Shell Ratio (SR) %**	**Filament length (m)**
M-Dch	18.36±0.01*	1.57±0.01*	0.32±0.01*	20.53±0.91*	859±2.00*
M-Cfl	18.11±0.02*	1.56±0.01*	0.31±0.01*	19.6±0.36*	807±2.07*
M-Mph	18.50±0.01*	1.57±0.01*	0.33±0.01*	20.73±0.3*	868±2.31*
M-Ame	19.24±0.01	1.59±0.01	0.34±0.01	21.85±0.32	951±2.08
M-Scy	19.25±0.01	1.59±0.01	0.34±0.01	21.84±0.41	952±1.15
N. bombycis	14.13±0.02*	1.50±0.01*	0.28±0.01*	18.62±0.66*	626 ±0.58*
Healthy Control	19.25±0.01	1.59±0.01	0.35±0.01	22.17±0.70	966±1.00

Data are expressed as Mean ±SD (n=5), *statistically significant (P<0.05) compared to healthy control larva by One way ANOVA using IBM SPSS20 Software.

Table 5.12**:** Effect of Microsporidian isolates (1×10^7 spores/ml) on larval and cocoon characters of the silkworm, *B. mori L.*

Microsporidian isolates (1×10^7 spores/ml)	**5 Mature larval weight**	**Cocoon Weight (g)**	**Shell Weight (g)**	**Shell Ratio (SR) %**	**Filament length (m)**
M-Dch	18.03± 0.01*	1.52±0.01*	0.30±0.02*	19.73±0.73*	841±2.31*
M-Cfl	17.73± 0.02*	1.50±0.01*	0.29±0.01*	19.15±0.31*	790±2.88*
M-Mph	18.16±0.02*	1.52±0.01*	0.30±0.01*	19.96±0.88*	843±1.53*
M-Ame	19.24 ± 0.01	159±0.01	0.34±0.01	21.80±0.34	951±2.08
M-Scy	19.24 ± 0.02	1.59±0.01	0.34±0.01	21.80±0.25	953±1.52
N. bombycis	13.97±0.02*	1.41±0.01*	0.26±0.01*	18.20±0.53*	608±1.73*
Healthy Control	19.25±0.01	1.59±0.01	0.35±0.01	22.17±0.70	966±1.00

Data are expressed as Mean ±SD (n=5), *statistically significant (P<0.05) compared to healthy control larva by One way ANOVA using IBM SPSS 20 Software.

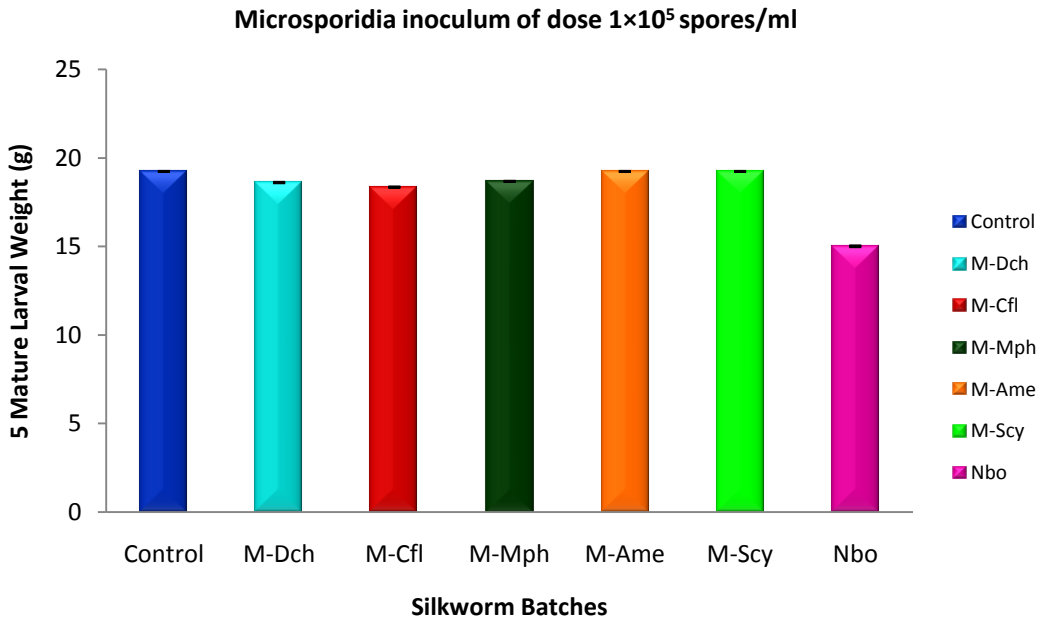


Figure 5.9: Graph showing weight of 5 mature larva in the silkworm batches inoculated with isolated microsporidia at dose concentration of 1×10^5 spores/ml.

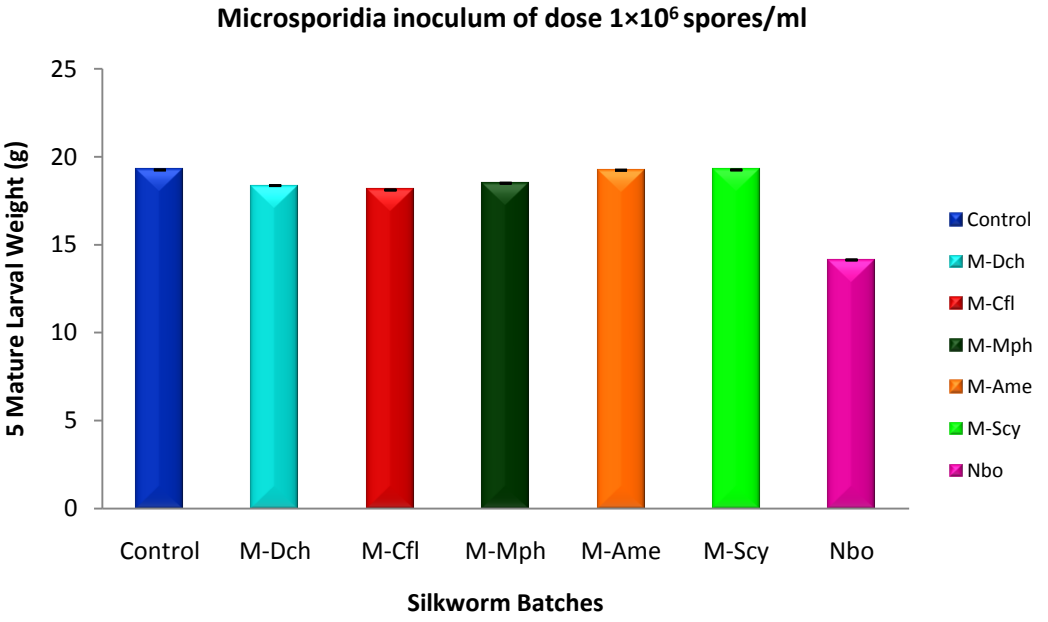


Figure 5.10: Graph showing weight of 5 mature larva in the silkworm batches inoculated with isolated microsporidia at dose concentration of 1×10^6 spores/ml.

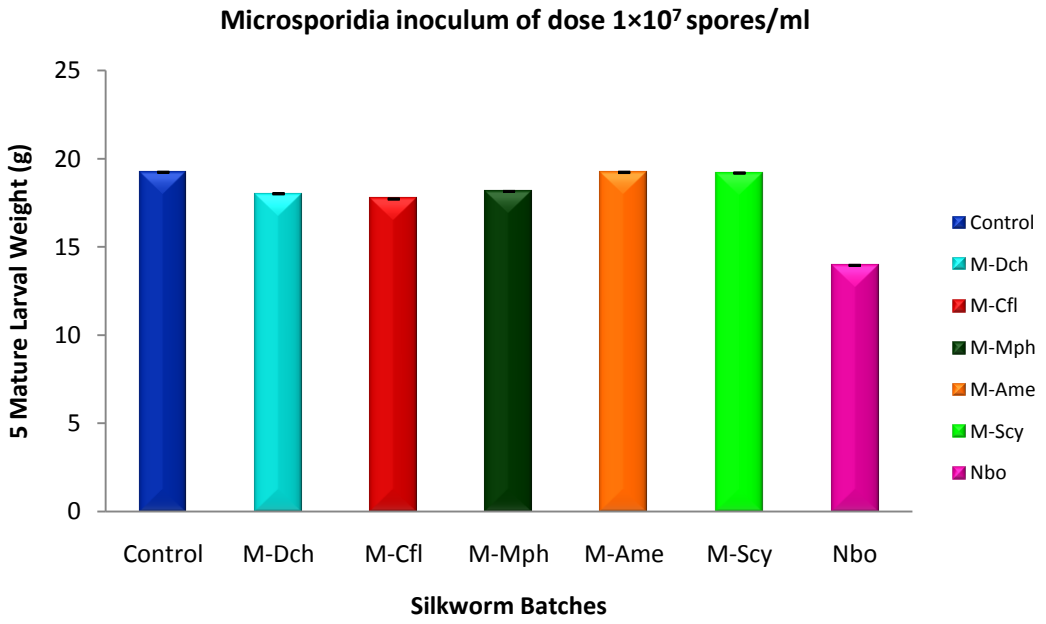


Figure 5.11: Graph showing weight of 5 mature larva in the silkworm batches inoculated with isolated microsporidia at dose concentration of 1×10^7 spores/ml.

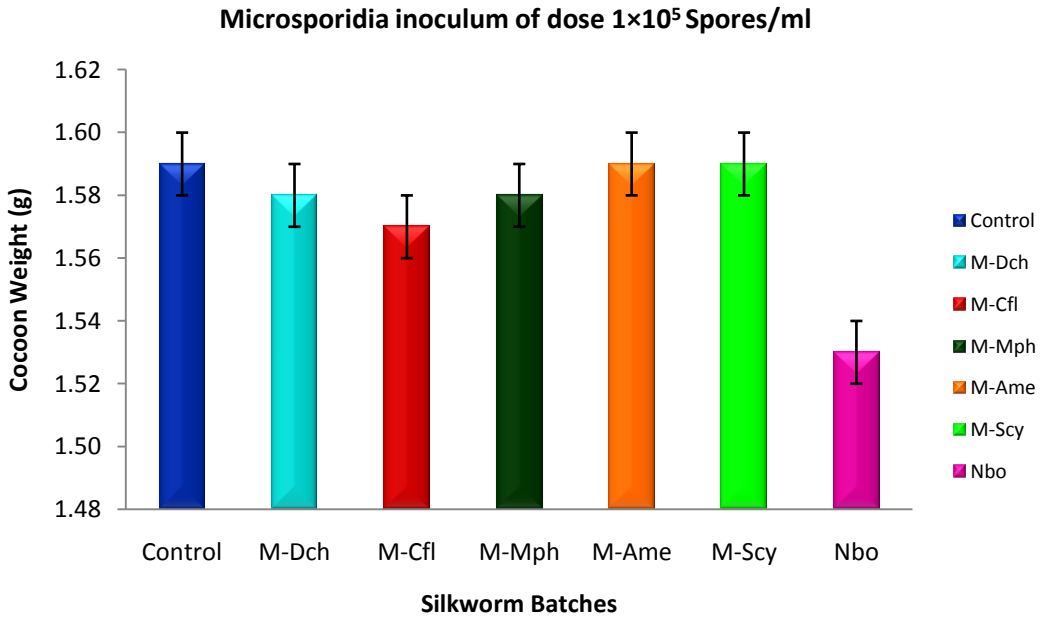


Figure 5.12: Graph showing cocoon weight of the silkworm batches inoculated with isolated microsporidia at dose concentration of 1×10^5 spores/ml.

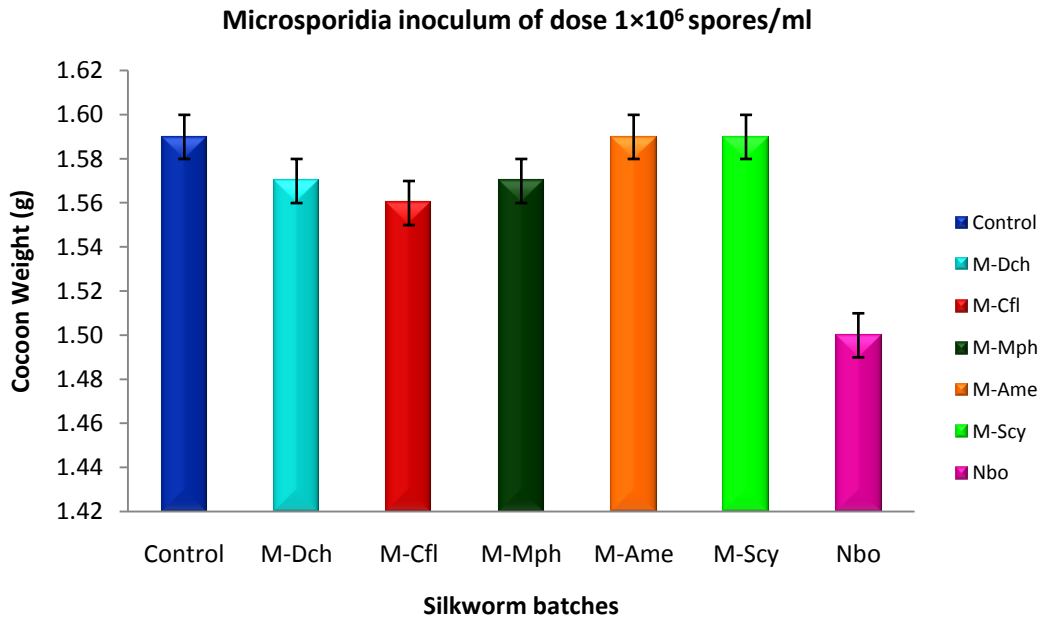


Figure 5.13: Graph showing cocoon weight of the silkworm batches inoculated with isolated microsporidia at dose concentration of 1×10^6 spores/ml.

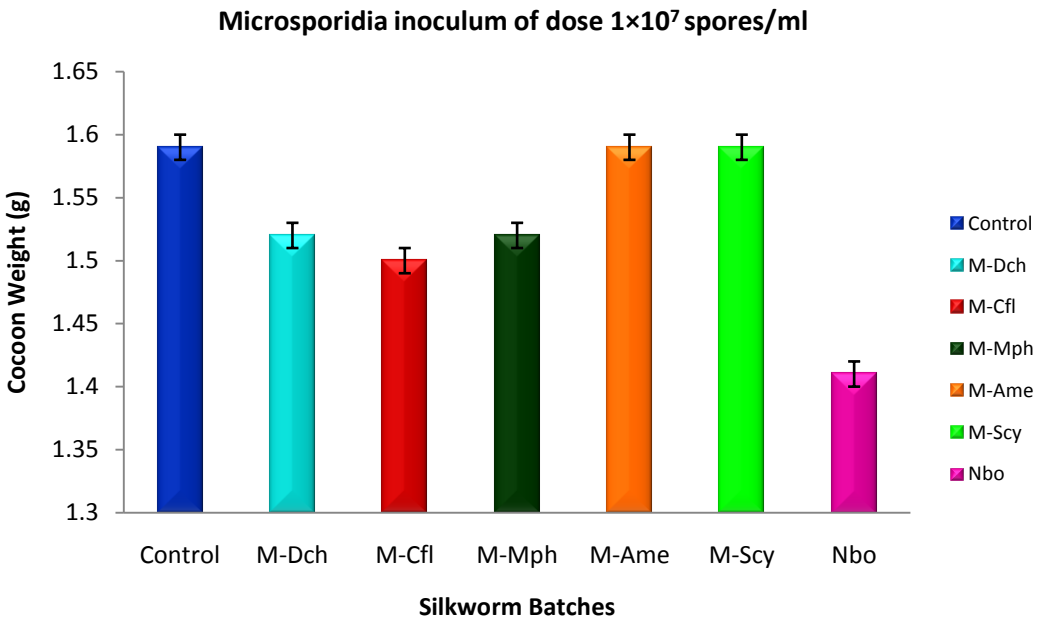


Figure 5.14: Graph showing cocoon weight (g) of the silkworm batches inoculated with isolated microsporidia at dose concentration of 1×10^7 spores/ml.

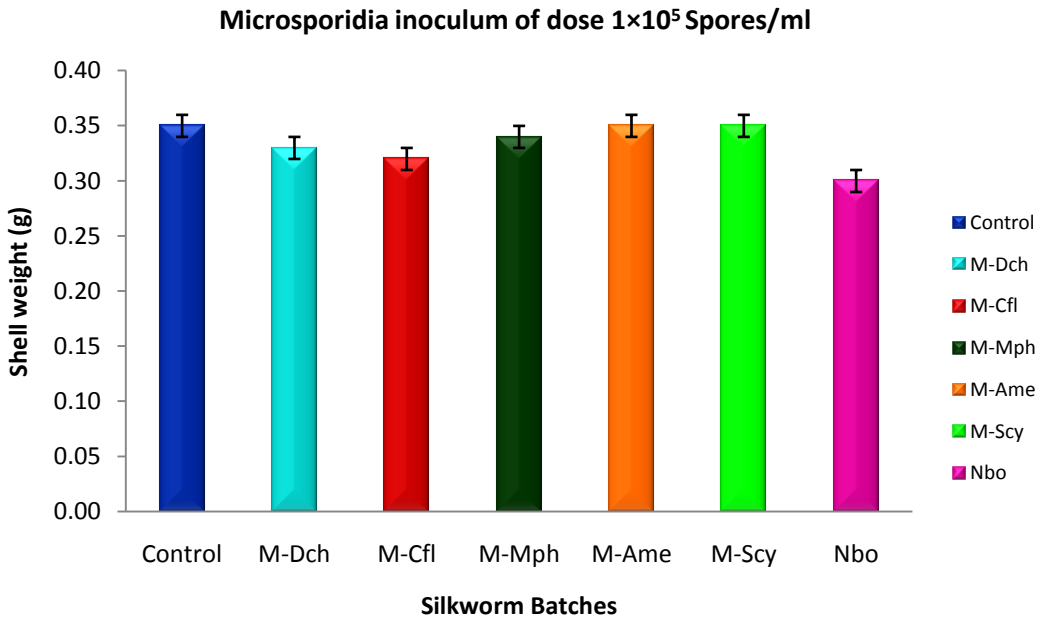


Figure 5.15: Graph showing shell weight (g) of the silkworm batches inoculated with isolated microsporidia at dose concentration of 1×10^5 spores/ml.

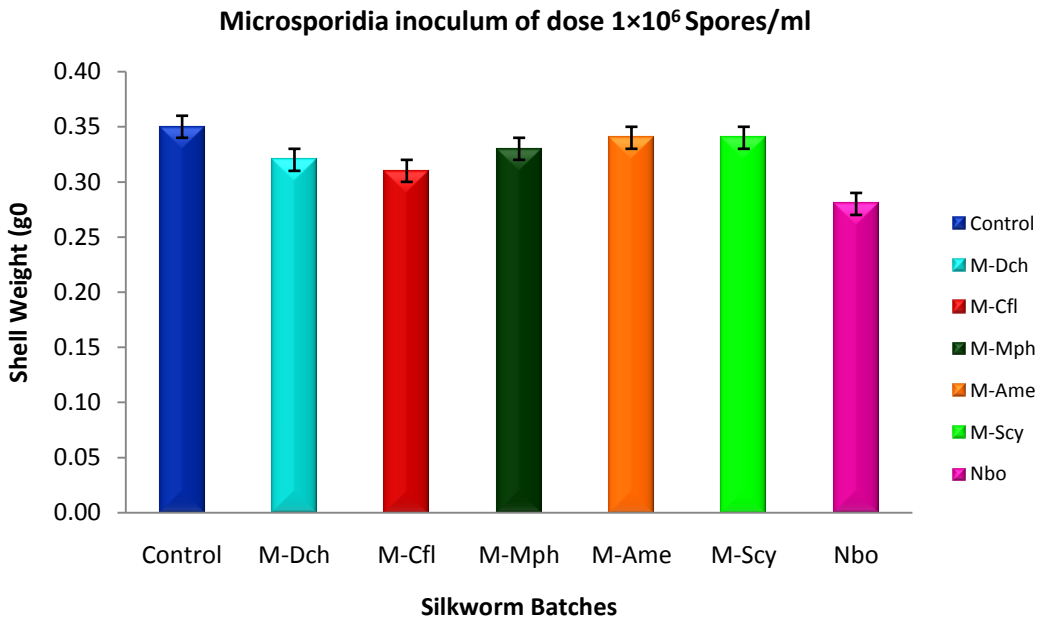


Figure 5.16: Graph showing shell weight (g) of the silkworm batches inoculated with isolated microsporidia at dose concentration of 1×10^6 spores/ml.

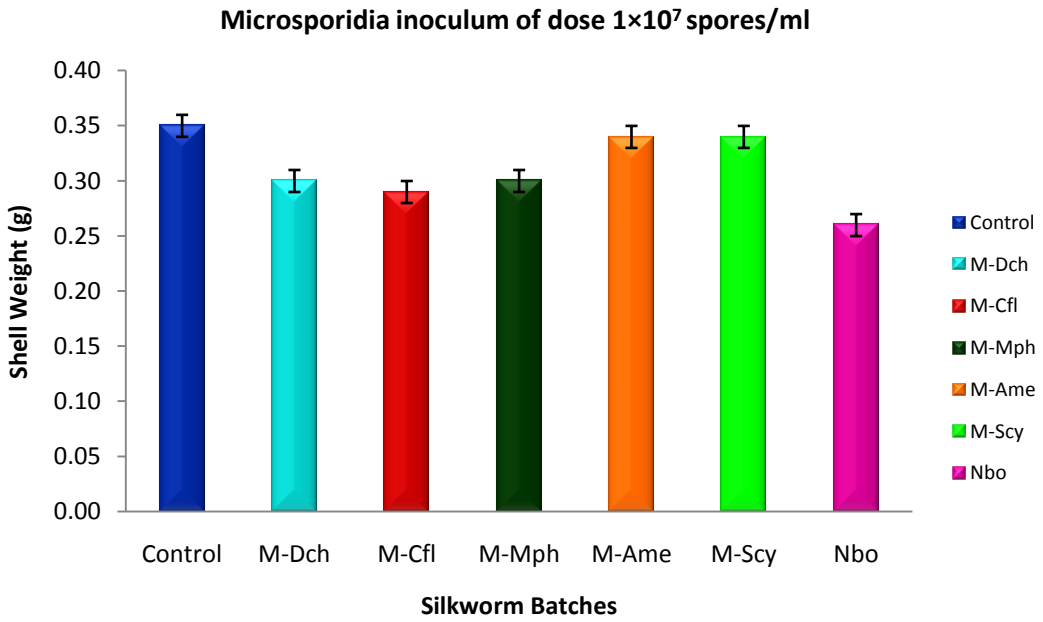


Figure 5.17: Graph showing shell weight (g) of the silkworm batches inoculated with isolated microsporidia at dose concentration of 1×10^7 spores/ml.

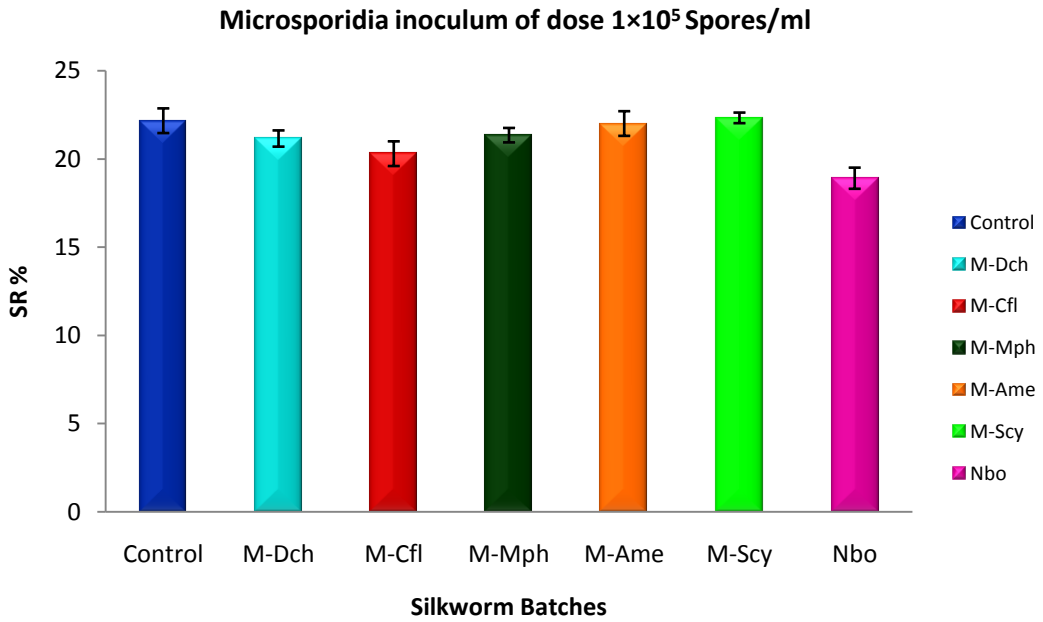


Figure 5.18: Graph showing Shell Ratio percentage (SR %) of the silkworm batches inoculated with isolated microsporidia at dose concentration of 1×10^5 spores/ml.

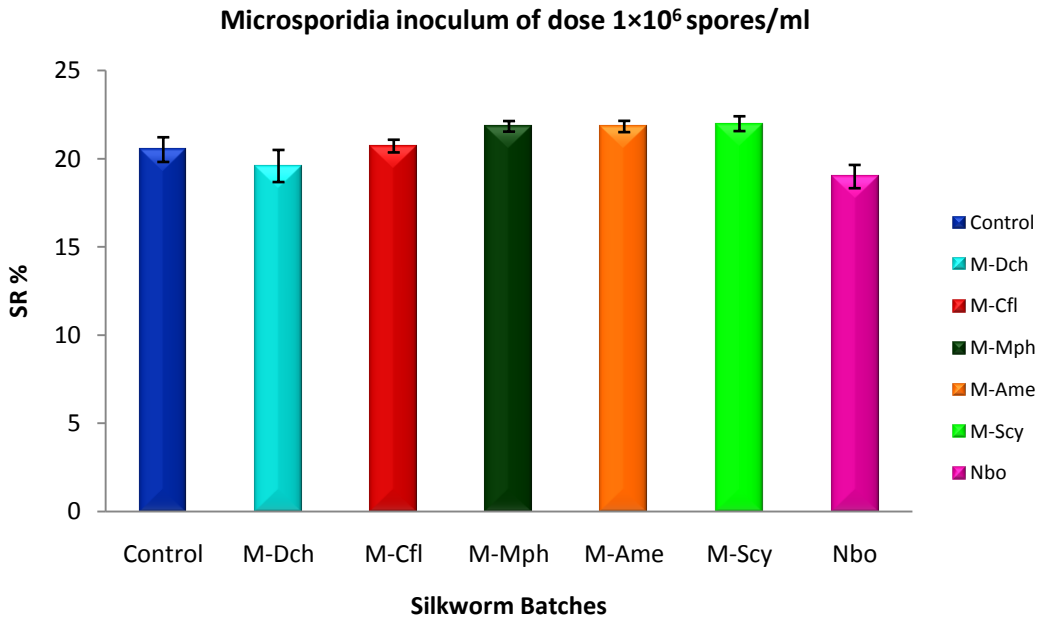


Figure 5.19: Graph showing Shell Ratio percentage (SR %) of the silkworm batches inoculated with isolated microsporidia at dose concentration of 1×10^6 spores/ml.

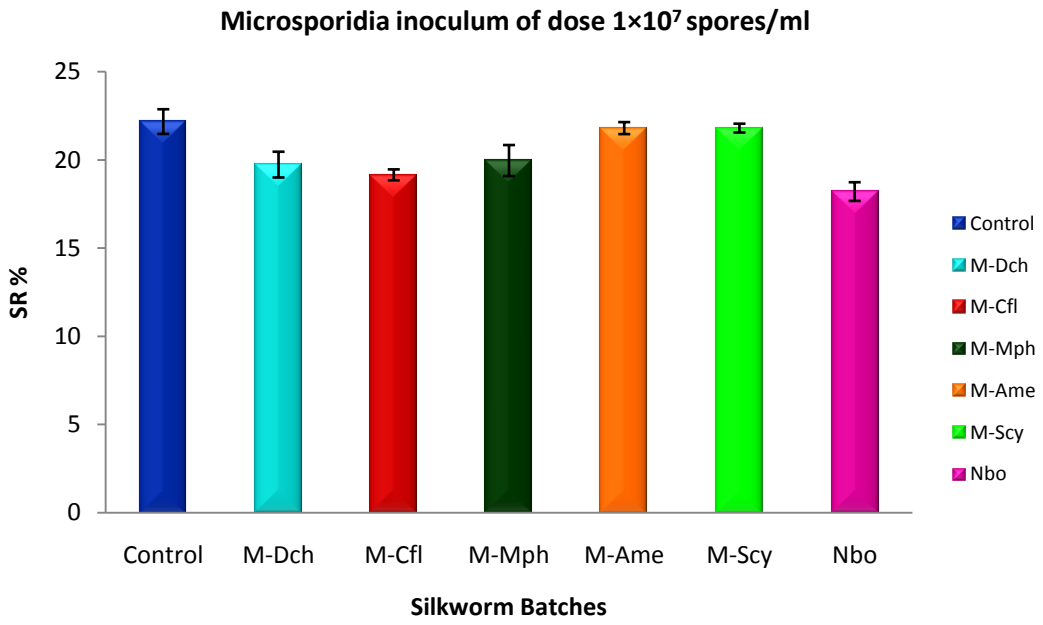


Figure 5.20: Graph showing Shell Ratio percentage (SR %) of the silkworm batches inoculated with isolated microsporidia at dose concentration of 1×10^7 spores/ml.

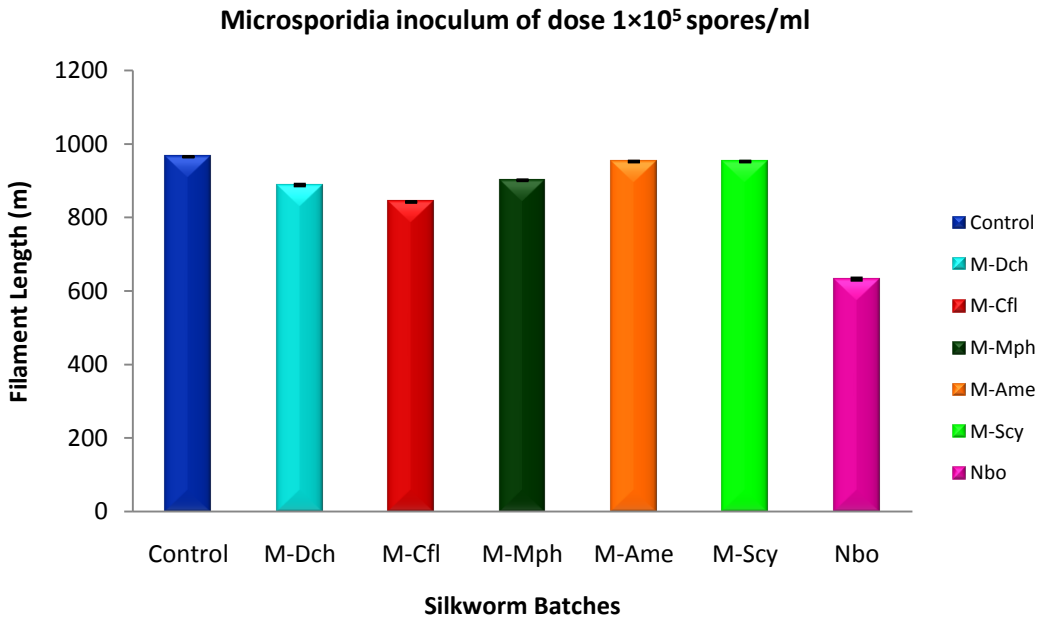


Figure 5.21: Graph showing Filament length (m) of the silkworm batches inoculated with isolated microsporidia at dose concentration of 1×10^5 spores/ml.

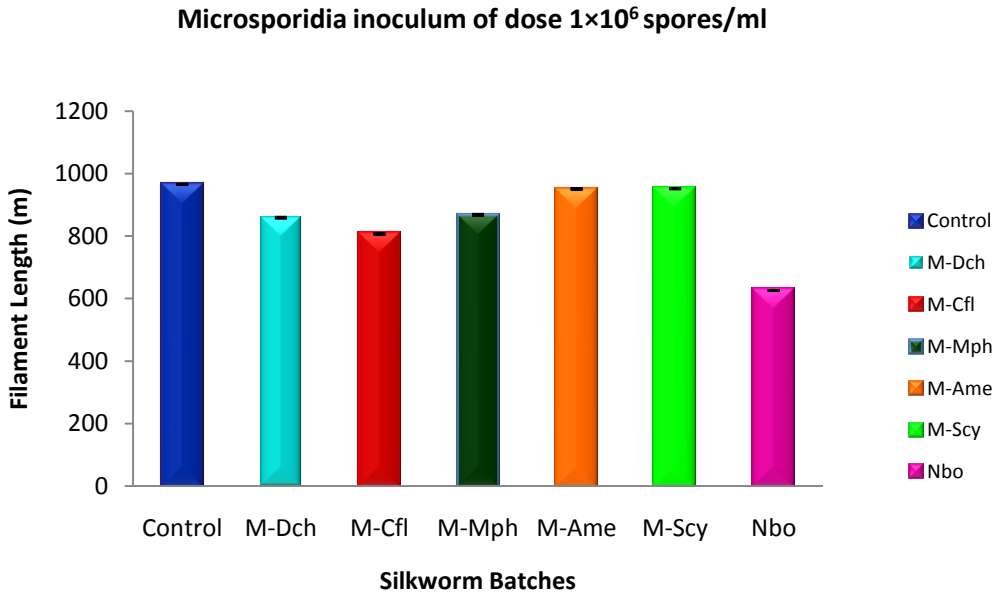


Figure 5.22: Graph showing Filament length (m) of the silkworm batches inoculated with isolated microsporidia at dose concentration of 1×10^6 spores/ml.

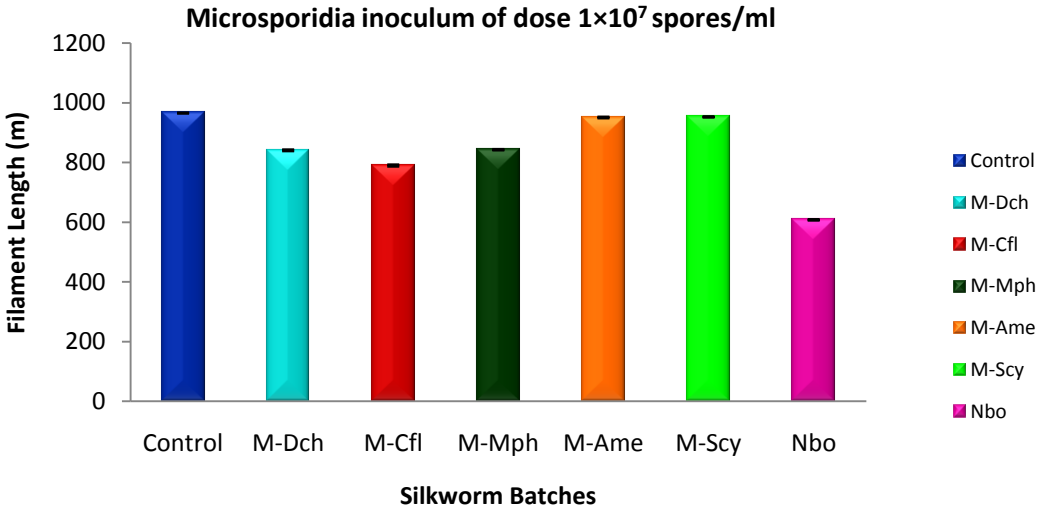


Figure 5.23: Graph showing Filament length (m) of the silkworm batches inoculated with isolated microsporidia at dose concentration of 1×10^7 spores/ml.

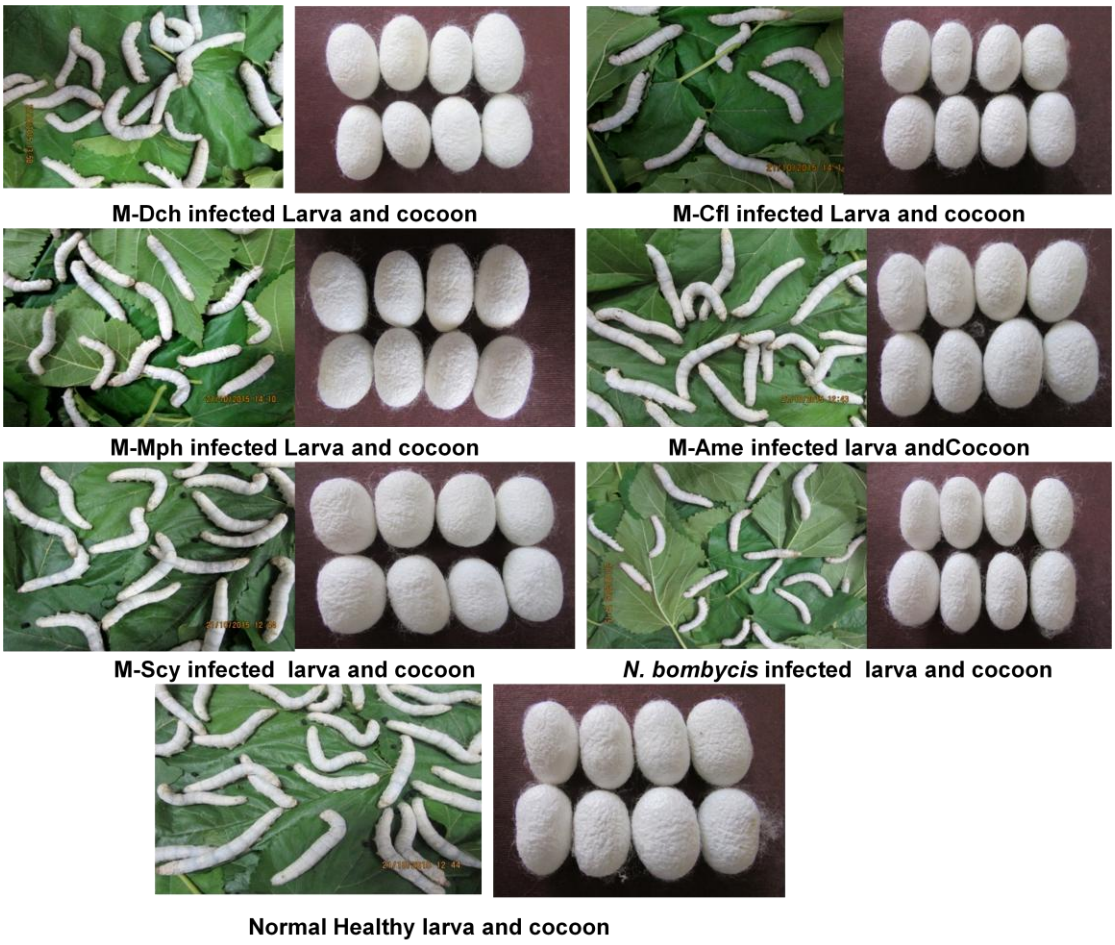
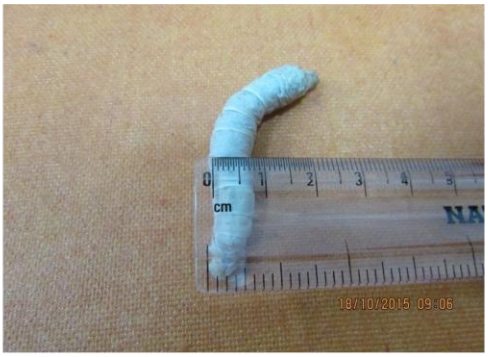


Figure 5.24: Images of silkworm, *B. mori* larva and cocoon from the experimental and healthy group

Measuring length of larva



Measuring width of larva

Measuring weight of cocoon

Healthy *B. mori*

B. mori Infected with microsporidia

Figure 5.25: Images showing the measurement of *B.mori* larval length, width and weight and a healthy and an infected *B. mori* silkmoth

5.3 DISCUSSION

Mulberry is the sole food plant for the silkworm, *B. mori* L. which is frequented by a number of insects either for food or for a casual visit from nearby agricultural crops. A number of workers have reported that these pests harbour different types of microsporidia which are cross infective to the mulberry silkworm *B. mori* L. (Kishore *et al.*, 1994; Sharma *et al.*, 1989, 2003; Bashir and Sharma 2008; Singh *et al.*, 2008, Bhuvaneswari and Surendra Nath 2015a, 2015b). Keeping in view the above, the present study was undertaken to investigate the incidence of microsporidian infection in the insect pests collected from mulberry gardens and agricultural crop fields. In the present study, microsporidian infection was recorded in number of insect samples but only the microsporidia isolated from five insects were selected for detailed investigations on the basis of the easy availability of these insects as well as on the basis of their infection burdens. The five insects selected were *D.*

chrysippus, *C. florella*, *M. phedima*, *A. mellifera* and *S. cynthia ricini* and the microsporidia isolated from them were tentatively designated as M-Dch, M-Cfl, M-Mph, M-Ame and M-Scy respectively. The infectivity of these isolated microsporidia was tested against the mulberry silkworm *B. mori* L. (CSR2×CSR4 bivoltine breed).

The microsporidia commonly known to cause the nosemosis disease in *B. mori* L. is *N. bombycis* (Sprague, 1982). However, a literature survey indicated that microsporidiosis in *B. mori* L. was not only caused by the standard strain of *N. bombycis* but also by different species/strains of *Nosema* and also by other microsporidians (Kawarabatta, 2003). Further, greater emphasis on understanding the microsporidiosis was often made by the researchers because of the very destructive nature of the disease caused by microsporidia in the silkworm. Therefore, it was found to be very important that studies were carried out to investigate the cross infectivity of the microsporidia isolated from various mulberry and agricultural insect pests to the silkworm. In India, there are very few reports on studies conducted to assess the cross infectivity of microsporidia isolated from various mulberry and agricultural insect pests to the silkworm. In addition, to our knowledge, this is the first report of any such study carried out in Uttar Pradesh to study the prevalence of microsporidia in common insect pests of mulberry and other agricultural crops and the possibility of cross infection of the microsporidia isolated from them to the silkworm *B. mori* L.

In the present study, the microsporidia isolated from the selected insects were administered to the silkworm *B. mori* L. through the oral route by feeding the silkworms with contaminated mulberry leaf smeared with the isolated microsporidia. A comparison of the infectivity of the isolated microsporidia M-Dch, M-Cfl, M-Mph with that of *N. bombycis* indicated that the isolated microsporidia caused significantly less mortality in larval stages as compared to that caused by *N. bombycis*. However, the other two microsporidian isolates *viz.* M-Ame and M-Scy were unable to infect the silkworm because when the larva were inoculated with these microsporidia and checked, all were found to be free of the microsporidian infection. Further, it was found that the isolated microsporidia adversely affected the economic characters of the silkworm. The larval length, larval width and larval weight of the silkworms were severely affected by the infection with the isolated microsporidia. The cocoon weight,

shell weight, shell ratio percentage (SR %) in the resultant cocoons formed by the infected larvae also showed a reduction when compared with healthy control, however, in all the cases the effect on silkworm due to the isolated microsporidia wa lesser than the effect caused by standard strain *N. bombycis*.

The highest larval mortality was observed in the *N. bombycis* inoculated batches of silkworm followed by M-Cfl, M-Dch, and M-Mph inoculated batches of silkworm. It was recorded that increase in dose concentration of microsporidia (M-Dch, M-Cfl, M-Mph and *N. bombycis*) spores increased the larval mortality which suggested that mortality rate was dose dependent. In M-Dch inoculated silkworm batches, the larval mortality increased from 11% to 35% as the concentration of dose increased from 1×10^5 spores/ml to 1×10^7 spores/ml where as in M-Mph inoculated batches, the larval mortality increased from 9% to 31% as the dose of M-Mph increased from 1×10^5 spores/ml to 1×10^7 spores/ml. Similarly, in the M-Cfl inoculated silkworm batches, the larval mortality increased from 12% to 40% as the concentration of dose increased from 1×10^5 spores/ml to 1×10^7 spores/ml where as in *N. bombycis* inoculated batches, the larval mortality increased from 21% to 81% as the dose of *N. bombycis* increased from 1×10^5 spores/ml to 1×10^7 spores/ml. The results were amply supported by a number of studies conducted by various other workers who have also concluded that larval mortality was always dose dependent (Choi *et al.*, 2002; Sharma *et al.*, 2014; Bhuvaneswari and Nath 2015a, 2015b). The silkworm larval mortality due to *N. bombycis* infection increased from 11.33% to 50.67% as the inoculum doses were increased from 1×10^3 spores/ml to 1×10^6 spores/ml (Sharma *et al.*, 2014). Choi *et al.* (2002) isolated microsporidia from cabbage white butterfly and inoculated to second instar *Pieris* larvae at a dosage of 1×10^8 spores/ml. They reported that the infection resulted in death of all larvae prior to adult eclosion, whereas at the lower spore dosage of 1×10^7 spores/ml,these workers reported that a few adults successfully emerged and at 1×10^4 spores/ml, many individuals survived to adulthood and only a few of these adults were infected.

In the present investigation, the median lethal concentration (LC_{50}) of the M-Dch and M-Mph for larval mortality was calculated as 3.2×10^7spores/ml and 9.67×10^7 spores/ml at 16 days post inoculation in the respective batches. Further, the median lethal concentration (LC_{50}) of the M-Cfl and *N. bombycis* for larval mortality

was calculated as 2.69×10^7spores/ml and 1.1×10^6 spores/ml at 16 days post inoculation in the respective batches. The recorded LC50 values clearly suggested that the standard strain *N. bombycis* was more virulent to the silkworm followed by M-Cfl, M-Dch and M-Mph microsporidian spores. Some workers have also reported the LC_{50} values of microsporidia isolated from different other insect pests against the larval mortality of silkworm *B. mori* L. The LC_{50} value of microsporidia isolated from butterfly *Pieris rapae* (NIK-1Pr), *Catopsilia crocale* (NIK-1Cc), *Catopsilia pyranthe* (NIK-1Cpy), *Spilosoma oblique* (NIK-1So) and *Diaphania pulverulentalis* (NIK-1Dp) against larval mortality of *B. mori* at 15 days PI was reported as $1\times10^{7.5}$ spores/ml, $1\times10^{9.5}$ spores/ml, $1\times10^{6.7}$ spores/ml, $1\times10^{0.1}$ spores/ml and $1\times10^{8.9}$ spores/ml respectively (Bashir, 2008). Xing *et al.*, (2014) and Bashir (2008) have reported the LC_{50} value of *N. bombycis* for silkworm larval mortality as 0.85×10^5 spores/ml for 12 days PI and $1\times10^{5.6}$ spores/ml respectively for 15 days PI.

In the present investigation, the results showed that the infection due to the isolated microsporidia caused significant changes in the larval morphology. The progressive increases in larval length, width and weight were found to be significantly affected in the inoculated batches as compared to the healthy control batches. The reduction in larval length, width and larval weight of silkworm due to pebrine and other parasitic infection has also been reported by many workers in earlier studies (Baig, 1994; Nath *et al.*, 1990; Rath *et al.*, 2000; Geortz *et al.*, 2004).

In the present study, it was observed that the isolated microsporidia significantly affected the cocoon characters *viz.* cocoon weight, shell weight, shell ratio % and filament length but to a lesser extent as compared to the *N. bombycis* inoculated batches of silkworm. Literature survey has clearly indicated that silk from the cocoons of pebrine infected larvae was inferior in strength and also the thickness of the silk was not uniform as compared to the healthy cocoon from control batches (Steinhaus, 1949). Jameson (1922) and Ghosh (1949) have also reported that pebrine infected silkworms spin flimsy and poor quality cocoons. Similar findings have also been reported in a recent study wherein the impact of a microsporidian (Lbms) isolated from a silkworm breed of North East Indian origin on economic characters of different bivoltine and multivoltine breeds of silkworm has been studied. According to the study, the microsporidian infection significantly lowered the larval weight,

single cocoon weight, shell weight and percent silk content of all the silkworm breeds studied (Bhat and Nataraju, 2005a). However, Sharma *et al.* (2014) reported that there were no significant differences in larval weights in the batches of silkworm larva inoculated with a new microsporidium and the batches inoculated with *N. bombycis* as compared to larval weight of healthy control batches These workers have however observed a significant reduction in the cocoon weight, shell weight and filament length of both the infected batches as compared to healthy batches of silkworm. Kudo (1931) reported that heavily infected larvae of *B. mori* did not spin cocoons and died, whereas mild infections allowed the larvae to spin cocoons.

5.4 CONCLUSION

The results of the present study, therefore, establish that a number of microsporidian strains are harboured by many insects and that these infections of the insects can cross infect the mulberry silkworm *B. mori* L. This may also be a possible explaination for the sudden and sporadic outbreaks of pebrine disease from time to time in sericultural areas in India. The present study concludes that out of five different microsporidia isolated from insect pests collected from mulberry gardens and other agricultural fields, three microsporidia showed considerable infectivity to the silkworm *B. mori* L. These microsporidia, therefore, constitute a potential threat of gaining entry into silkworm rearing and perpetuate the infection despite routine care taken in mother moth examination and sanitations. The present study also underlines the need for further detailed studies on the population dynamics of various insect pests of mulberry and other agricultural crops and their role in the outbreak of microsporidian disease in silkworm.

Summary

SUMMARY

Parasitology is a growing science and in order to control and fight parasitic diseases and infections, a quick and reliable diagnosis of the infection agent is necessary. The insects comprise the largest group of organisms which are vulnerable to various diseases caused by microbes such as viruses, bacteria, fungi and protozoans. Microsporidia are the most diversified fungal entomopathogens that can infect both vertebrate and invertebrate hosts however, most prevalent in insects. The parasite causes a dreaded disease in the host body called microsporidiosis.

Microsporidia are unicellular organisms producing an environmentally resistant spore, characterized by the presence of unique extrusion apparatus housing a highly coiled polar filament and one or more nuclei. Infection occurs when a susceptible insect ingests the environmental spores of a microsporidium. They germinate in the mid gut under the influence of certain ions, alkaline pH and digestive enzymes and infect the epithelial cells and cause chronic infections which can be sometimes lethal. Many species of microsporidia, besides the normal mode of horizontal transmission, are even transmitted vertically from the parents to the progeny.

Furthermore, microsporidia are regarded as efficient regulators of insect populations in nature. Several published reports authenticate this fact and therefore, studies in this track assume greater significance. Microsporidia have been studied for over 160 years, but yet many doubts persist regarding their life cycle and biological features, as well as their taxonomy and phylogeny. Therefore, careful studies are indispensable for the correct identification of new and existing species.

The extensive work has been carried out in several countries on microsporidia infecting the insect species, but it is largely unexplored in India. In India, majority of the studies were carried out on *Nosema bombycis* or other *Nosema* sp. related to silkworm by sericulture personnel. Therefore, in India, there is much scope to study the diversity of microsporidia in the natural insect population. This is the first study on microsporidia being carried out in Lucknow region of Uttar Pradesh. The present study was carried out to gather information about the presence of microsporidia among mulberry and other agricultural insect pests. Therefore, the present study was

carried out to investigate the prevalence of microsporidian infection in insect pests of mulberry and other agricultural crops and to study their morphology, ultrastructure and infectivity to mulberry silkworm, *B. mori* L.

The first chapter of the thesis gives the relevant introduction of the research topic depicting the aims and objectives of the proposed study.

The second chapter includes the review of the literature available from different sources (printed/online).

The third chapter of the thesis gives a brief idea about the distribution and prevalence percentage of microsporidian infection in the insect population commonly inhabiting the mulberry and agricultural fields. There is less literature available about the prevalence of microsporidian parasites in insects. In the present study, a total of 34 different insect species were collected, being categorized into five insect orders *viz.* Lepidoptera, Hymenoptera, Orthoptera, Coleoptera and Hemiptera. The lepidopteran insect species were frequently available in the study sites as compared to other insects. Further, it was observed from literature survey that the lepidopteran and hymenopteran insects are more susceptible to microsporidian infections. Keeping in view the easy availability of the insect species and the intensity of microsporidian infection in the respective host species, four lepidopteran insects (*D. chrysippus, C. florella, M. phedima, S. cynthia ricini*) and one hymenopteran insect (*A. mellifera*) were selected for the detailed investigations. In the present study, among all the insects collected, highest prevalence percentage of microsporidian infection was recorded in *Apis mellifera* (52.33%). Among lepidopteran insect species investigated in the present study maximum prevalence percentage of microsporidian infection was recorded in *D.chrysippus* (33.51%) followed by *S. cynthia ricini* (32.69%), *M. phedima* (25.31%), *C. florella* (24.42%), *C. pyranthe* (13.88%), *D. genutia* (11.11%), *M. leda leda* (6%). Among the orthopteran and coleopteran insects collected during the present investigation, some of the insects were found positive with microsporidian infection but the intensity of the infection was very low. Therefore, these insects were not selected but for the brief study of microsporidia infecting them. From the present study it can be concluded that microsporidian infection was prevalent in insect population of Lucknow region in India. However, the infections often go unnoticed and their role in population dynamics of insects is often not recognized because

microsporidia do not cause dramatic epizootics such as those caused by fungi and viruses.

The fourth chapter describes the microscopic study of microsporidian spores isolated from insect pests of mulberry and other agricultural crops. The live micosporidian spores were easily detected by their characteristic Brownian movement. The light microscopy revealed that the five different microsporidia, tentatively named M-Dch, M-Cfl, M-Mph, M-Ame, M-Scy were oval in shape with variable sizes. The live microsporidian spores showed translucent properties with high refractivity whereas the germinated spores were easily distinguished from the live spores and observed as black empty spores under phase contrast microscope.

The sizes of the fresh microsporidia were larger than the fixed microsporidia. The size (Length × Width) of the fresh microsporidia, M-Dch, M-Cfl, M-Mph, M-Ame and M-Scy were measured as 4.06±0.12 µm × 2.29±0.08 µm, 4.32±0.11 µm × 2.33±0.06 µm, 5.35±0.09 µm × 3.26±0.11 µm, 4.35±0.10 µm × 2.80±0.07 µm and 3.52±0.12 µm × 2.22±0.10 µm respectively; whereas the size of Nbo spores were measured as 3.27±0.10 µm × 2.09±0.07 µm. Again, it was observed that the mean sizes of the above five microsporidia were smaller than that of the spore size of *N. bombycis*.

Under SEM, the textures of the spore surface were studied. The exospore wall of the M-Dch, M-Cfl, M-Mph and M-Scy spores were smooth whereas the exospores of M-Ame spores were ornamented with ridges and furrows. The meront and spore stages of the microsporidia were clearly distinguished as meronts were rounded in shape and spore stages were oval in shape. Further, since light microscopic techniques have proved inadequate to differentiate between species of similar appearance, ultrastructural methods have become more and more important (Hazard and Oldacre, 1975; Weiser, 1977). For differentiation of microsporidian species, the attention of taxonomists has been increasingly drawn to the surface structures of the spores.

The TEM ultrastructural features of M-Dch, M-Cfl, M-Scy and M-Ame spores confirmed that they belonged to the genus *Nosema* whereas the ultrastructure of M-Mph confirmed that it belonged to the genus *Larssoniella*. The microsporidian species were identified after the descriptions of Weiser (1961) and Maurand (1973) and by

using the additional observations of Maurand (1975) and Larsson (1999). The microsporidia under the genus *Nosema* are characterized by the production of spores with walls consisting of an electron dense exospore and an electron lucent endospore, polar filament coils, and diplokaryotic nuclei (Franzen & Müller, 1999; Huang *et al.*, 2007). The microsporidia *N. bombycis* from *B. mori* has 11-14 coils with the angle of tilt on most anterior to posterior coil is 62° and 61°. M-Dch and M-Cfl spore possessed 11 and 14 polar filament coils respectively. In M-DCh spore, the angle of tilt of the most anterior and most posterior polar filament coil to the spore axis were measured as 42° and 90° whereas in case of M-Cfl spore, the angle of tilt of the most anterior to posterior polar filament coil to the spore axis were measured as 90°. M-Ame spore possessed 21 polar filament coils arranged in two rows with an angle of tilt of 55°. The M-Scy spore contained 12 polar filament coils with an angle of tilt of the most anterior to posterior coil to that of the spore axis being measured as 40°. Further, the exospores and endospore walls of M-Scy spore were measured as 22 nm and 120 nm respectively. The M-Mph spore described in the present investigation possessed a single nucleus and 11 polar tube coils with an angle of tilt of the anterior coil as 25° and therefore, shared ultrastructural characteristics similar to that of the genus *Larssoniella*.

Microsporidian infections in insects are generally chronic, causing pathogenic effect on host and reduce their fecundity and life spans. Microsporidia are highly prevalent in insect population and they are diagnosed under the microscope by their transluscent properties and Brownian movement. However, the shape, size, spore surface and number of polar filament and their angle of tilt differ for each microsporidian species. The SEM study showed that the exospores of M-Dch, M-Cfl, M-Mph and M-Scy were smooth whereas exospores of M-Ame spore was ornamented with ridges and furrows. From the ultrastructure, the microsporidium M-Dch, M-Cfl, M-Ame and M-Scy were identified as genus *Nosema* Naegeli, 1857 whereas the microsporidium M-Mph was identified as genus *Larssoniella*. From the present investigation, it can be concluded that microsporidian parasites belonging to genus *Nosema* are common in insect pests. The microsporidian infection leads to greater economic losses to a country when they infect the insect species like honey bee, silkworm etc. They are considered to be more useful as long-term regulators of

pests and contribute towards the prevention and/or suppression of pest outbreaks. In spite of the role of microsporidia in the natural biocontrol of insect populations, relatively limited informations are available about the diversity of microsporidia. Therefore, the present study provides a much needed input to the information about the morphology and ultrastructure of the microsporidian parasites harbouring in insect populations.

The fifth chapter describes the studies on the infectivity of the microsporidian spores isolated from insect pests of mulberry and other agricultural crops to the silkworm *Bombyx mori* L. In this chapter, the infectivity of the microsporidian isolates M-Dch, M-Cfl, M-Mph, M-Ame, M-Scy and *N. bombycis* (Nbo) were checked against mulberry silkworm *B. mori*. The larval mortality due to microsporidian infection was not recorded in the silkworm batches inoculated with M-Ame and M-Scy spores where as the dead larvae in the silkworm batches inoculated with M-Dch, M-Cfl, M-Mph and *N. bombycis* (Nbo) were found positive for the infection.

At 16 days post inoculation, the LC_{50} value of M-Dch, M-Cfl and M-Mph spores against larval mortality of *B. mori* were calculated as 3.2×10^7 spores/ml, 2.69×10^8 spores/ml and 9.67×10^7 spores/ml respectively whereas the LC_{50} value of *N. bombycis* (Nbo) against larval mortality of *B. mori* was calculated as 1.1×10^6 spores/ml. The results indicate that among the isolated microsporidia, M-Cfl was more virulent to silkworm *B. mori* followed by M-Dch and M-Mph spores but were found to be less virulent than *N. bombycis* as the highest larval mortality was recorded in silkworm batches inoculated with *N. bombycis* spores.

Studies on the impact of infection on larval characteristics showed that there was no significant reduction in larval length of silkworm in the batches inoculated with microsporidia M-Ame and M-Scy. It was also observed that these microsporidia were not pathogenic to the silkworm *B. mori* L. In the present investigation, the infection due to the isolated microsporidia M-Dch, M-Cfl and M-Mph caused significant changes in the larval morphology. The progressive increases in larval length, width and weight were found to be significantly affected in the inoculated batches as compared to the healthy control batches. Again, it was observed that these microsporidia significantly adversely affected the cocoon characters *viz.* cocoon

weight, shell weight, shell ratio % and filament length but to a lesser extent as compared to the effect of *N. bombycis* inoculated batches of silkworm.

The present study, therefore, establishes that a number of microsporidian strains are harboured by many insects and these can also cause cross infection in silkworm. This may also explain the sudden and sporadic outbreaks of pebrine disease from time-to-time in the sericultural areas in India. The study concludes that out of five different microsporidia isolated from the insect pests collected from mulberry gardens and other agricultural fields, three microsporidia showed considerable infectivity to the silkworm *B. mori* L. These microsporidia, therefore, constitute a potential threat of gaining entry into silkworm rearing and perpetuating the infection despite routine care taken in mother moth examination and sanitations.

The present study revealed that the five microsporidia (M-Dch, M-Cfl, M-Mph, M-Scy and M-Ame) isolated from the insect pests of mulberry and some other agricultural crops were cross infective to silkworm and possessed the characteristic features resembling umpteen microsporidia on one hand, but differed from the standard microsporidian strain *N. bombycis* infecting the silkworm, *B. mori* L. on the other hand in terms of their ultrastructural features. The microsporidia M-Dch, M-Cfl and M-Mph showed infectivity to the mulberry silkworm, *B. mori* L. through oral portals.

The present study underlines a detailed study on the prevalence, morphology and ultrastructure of the microsporidian infections in various insect pests of mulberry and other agricultural crops which may also contribute to the outbreak of microsporidian disease in silkworm. It can be concluded that the insect pests associated with mulberry and other agricultural crops harbor a number of microsporidian strains that are also cross-infective to the silkworm. However, these microsporidia are less pathogenic to silkworm and are not transmitted by transovarial means but constitute a potential threat to the silkworm rearing through contaminated mulberry leaf.

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Appendix

APPENDIX

PAPERS PUBLISHED IN JOURNALS

S.No	Title of the paper	Authors	Journal Name/ Volume (issue)/ Page Number	Year
1	Seasonal prevalence of microsporidian infection in the plain tiger Butterfly, *Danaus chrysippus*	Mukta Mayee Kumbhar, Kamal Jaiswal and Suman Mishra	Journal of Environment and Biosciences, Vol. 31 (1): 219-221 ISSN 0973-6913 (Print), ISSN 0976-3384 (On Line), NAAS Rating- 4.43	2017
2	Cross-infectivity of microsporidia (M-Cfl) isolated from *Catopsilia florella* on larval and cocoon Parameters of the silkworm, *Bombyx mori* L.	Mukta Mayee Kumbhar, Kamal Jaiswal and Suman Mishra	Proceedings of the Zoological Society of India, Vol 16 (1): 85-94, ISSN 0972-6683, NAAS Rating- 4.42	2017

CHAPTER PUBLISHED IN BOOK

S.No.	Title of the Chapter, Authors, Page Nos.	Book Title, Editor & Publisher	ISSN/ISBN No.
1	Screening of Hymenopteran Insects for Microsporidian Infection. Mukta Mayee Kumbhar and Suman Mishra pp. 91-98	**Trends in Biodiversity: Floral, faunal and ecological aspects** Editors- RC Sobti, Kamal Jaiswal, Suman Mishra Published by Narendra Publishing House, New Delhi.	ISBN: 978-93-84337-65-0

PAPERS PRESENTED IN CONFERENCES/ SYMPOSIA/SEMINARS/WORKSHOPS

Sl. No.	Authors & Title of the Paper	Conference/Symposium/ Workshop	Date
1.	Mukta Mayee Kumbhar and Luna Samanta Effect of industrial effluent in N*otopterus notopterus* of Mahanadi river collected from upstream and downstream of Choudwar industrial area. Abstract No.- YP-3, pp- 64	National Workshop on Capacity Building in Disease Control & Sustenance & Mini Symposium on Parasitic Diseases, Organized by Department of Zoology, University of Allahabad, Allahabad, U.P.	5^{th}-7^{th} November, 2012
2.	Mukta Mayee Kumbhar Climate Change, Its Implications and Management Strategies	Seminar on "Environment, Education & Society" Organized by Department of Environmental Science, B.B. Ambedkar University, Lucknow	5^{th} June, 2013
3.	Mukta Mayee Kumbhar and Luna Samanta Modulatory effect of charge chrome industry effluents and induced oxidative stress indices in *Notopterus notopterus*.	National Workshop on Biomolecular Parasitology and Resource Sustainability Organized by University of Allahabad, Allahabad	3^{rd}-5^{th} December, 2013
4.	Mukta Mayee Kumbhar and Suman Mishra Different Modes of Transmission of Microsporidia in Insects. Abstract No.- PHS, pp- LS-15.	2^{nd} Lucknow Science Congress Organized by B.B. Ambedkar University, Lucknow	27^{th}-28^{th} March, 2014
5.	Mukta Mayee Kumbhar, Narendra Awasthi and Suman Mishra.	International Conference on Faunal Biodiversity and their Conservational strategies.	22^{nd}-23^{rd} March, 2014

	Studies on the Paramphistome Parasites of Goat. Abstract No.- BEP43, pp-77	Organized by Lucknow University, Lucknow	
6.	Mukta Mayee Kumbhar and Suman Mishra Screening of Hymenopteran Insects for Microsporidian Infection Abstract No.- PP-117, pp-185	International symposium on "Biodiversity: Status, Utilization and Impact of Challenging Climatic Conditions, Organized by B.B. Ambedkar University, Lucknow in collaboration with Indian Academy of Environmental Sciences, Haridwar, Uttarakhand	30th-31st October, 2014
7.	Mukta Mayee Kumbhar and Suman Mishra Polar Tube: A Diagnostic Tool for Characterization of Microsporidia Abstract No.-176, pp-120	National Seminar on "Threats to Biodiversity and Ecosystems: Impact of Developmental Projects and Climate Change and 25th National Congress of Zoology" Organized by Gurukula Kangri Vishwavidyalaya, Haridwar	17th-19th November, 2014
8.	Mukta Mayee Kumbhar and Suman Mishra Detection of Microsporidia in the Brinjal Leaf Roller *Autoba olivacea* Walker, 1858 Abstract No.- OP-20. pp- 85	26th All India Congress of Zoology and International conferences on Innovation in Animal Sciences For food security, Health security and Livelihood-2015 Organized by Department of Applied Animal Sciences, B.B. Ambedkar University, Lucknow	29th-31st October, 2015
9.	Mukta Mayee Kumbhar and Suman Mishra Diversity of Microsporidian Parasites in the Ecosystems: A Review Abstract No.-OP-11, pp- 29	3rd Lucknow Science Congress and National Conference on "Science for Society: An Interdisciplinary Approach". Organized by B.B. Ambedkar University, Lucknow	31st October to 2nd November, 2015
10.	Mukta Mayee Kumbhar and Suman Mishra Genome Reduction in Microsporidia	103rd Indian Science Congress, Organized by University of Mysore, Mysuru, Karnataka	3rd-7th January, 2016

	Abstract No.- EP-P-52, pp-153		
11.	Mukta Mayee Kumbhar and Suman Mishra Light microscopic study of microsporidia isolated from striped tiger butterfly, *Danaus genutia* C.	Three-day seminar on the theme "Science & Technology for Specially Abled Person", organized by Department of Applied Animal Sciences, B.B. Ambedkar University, Lucknow in association with Zoological Society of India, Bodhgaya.	28th February to 2nd March, 2017
12.	Mukta Mayee Kumbhar, Kamal Jaiswal and Suman Mishra Study on the Microsporidia Infecting Western honey bee, *Apis mellifera* Abstract No.- ZGZ-8, pp- 8	International Symposium on Culture Based Fisheries in Inland Open waters & Satellite Symposium on Fish Immunology, 29th All India Congress of Zoology Organized by ICAR- Central Inland Fisheries Research Institute, Barrackpore, India	9th-11th June, 2017

CONFERENCES/ SYMPOSIA/ SEMINARS/ WORKSHOPS ATTENDED

S.No	Name of the Seminar/Conference / Symposia / Workshop, etc.	Organizer	Date
1.	International Conference on "Recent Trends in Climate Change Researches vis-à-vis Biodiversity"	Department of Animal Science, MJP Rohilkhand University, Bareilly	3rd-4th December, 2012
2.	Programme on "Wetlands in Biodiversity Conservation"	Department of Animal Sciences, B.B. Ambedkar University, Lucknow	2nd February, 2013
3.	National Workshop on "Crime Against Women: Legal Issues"	School for Home Sciences, B.B. Ambedkar University, Lucknow	7th March, 2013
4.	1st Lucknow Science Congress (LUSCON-2013)	B.B. Ambedkar University, Lucknow	20th-21st March, 2013
5.	One Day Workshop on "Enhancing Communication Skills of Students in Higher Education: Role of Libraries"	Information & Guidance Bureau & DLIS, B.B. Ambedkar University, Lucknow	22nd May, 2013

6.	Symposium on " Building an Ecologically Sustainable Society"	B.B. Ambedkar University, Lucknow	16th August, 2013
7.	One Day Seminar on "ICT In Higher Education: Need of the Hour"	Information & Guidance Bureau & DLIS, B.B. Ambedkar University, Lucknow	20th January, 2014
8.	103rd Indian Science Congress	University of Mysore, Mysuru, Karnataka	3rd-7th January, 2016

PARTICIPATION IN TRAINING PROGRAMMES/ WORKSHOPS

S.No	Name of the Workshop, etc.	Place	Date
1.	National Workshop on Capacity Building in Disease Control & Sustenance & Mini Symposium on Parasitic Diseases (CBDCS-2012)	Parasitology laboratory, Department of Zoology, University of Allahabad, Allahabad, U.P., India	5th-7th November, 2012
2.	National workshop on Biomolecular Parasitology and Resource Sustainability (BPRS-2013)	Parasitology laboratory, Department of Zoology, University of Allahabad, Allahabad, U.P., India	3th-5th December, 2013
3.	15 days training Programme on "Silkworm Pathology"	Central Sericultural Research & Training Institute (CSR&TI), Mysore, Central Silk Board, Ministry of Textiles, Govt. of India	10th-24th July, 2014
4.	Hands-on-Training on SEM, FTIR, FPLC and Ion Chromatography	B.B. Ambedkar University, Lucknow	18th-20th February, 2015
5.	12 days training Programme on "Morphological Characterization of Microsporidia"	Central Sericultural Research & Training Institute (CSR&TI), Berhampore, West Bengal, Central Silk Board, Ministry of Textiles, Govt. of India	20th June to 1st July, 2015

MEMBERSHIP OF PROFESSIONAL BODIES/ORGANIZATIONS

- Annual Membership, **Indian Academy of Environmental Science (2017-18)**
- Life Membership, **Zoological Society of India,** Membership No: M-67
- Life Membershi**p, Indian Science Congress Association,** Membership No: L2772**0**

FELLOWSHIP/ AWARD RECEIVED

- UGC Rajiv Gandhi National Fellowship 2010-11.
- Best poster presentation award received from Zoological Society of India on the occasion of 25th All India Congress of Zoology and National Seminar on "Threats to Biodiversity and Ecosystems: Impact of Developmental Projects and Climate Change". Organized by Department of Zoology and Environment Science, Gurukul Kangri Vishwavidyalaya, Haridwar, 17th-19th November, 2014.
- Received Young Scientist Gold Medal from Zoological Society of India on the occasion of 29th All India Congress of Zoology and International Symposium on Culture Based Fisheries in Inland Open waters & Satellite Symposium on Fish Immunology, Organized by ICAR- Central Inland Fisheries Research Institute, Barrackpore, West Bengal, 9th -11th June, 2017.