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LIGHT AND ELECTRON MICROSCOPY OF *Nosema ricini* (Microsporidia: Nosematidae), THE CAUSAL PATHOGEN OF PEBRINE DISEASE IN ERI SILKWORM: LIFE CYCLE AND CROSS-INFECTIVITY

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ABSTRACT

Pebrine is a deadly disease of silkworm causing serious damage in silkworm and is directly responsible for the decline of sericulture industry in many countries. The disease is caused by *Nosema ricini,* a pathogenic microsporidian (Microsporidia: Nosematidae) in eri silkworm, *Philosamia ricini* Boisd. This paper for the first time documents a detailed study on morphology and life cycle stages of *N. ricini* as observed under light, scanning and transmission electron microscopy. The mature spores of *N. ricini* were elliptical in shape, blunt or tapering at both ends and small in length (3.8±0.06 µm), breadth (2.6±0.03 µm) and volume (13.7 µm^3). Mature spore had hard spore wall and slight concavity or were tapered at both the ends of spore with smooth external morphology as seen under SEM. The main components of spore viz., sporoplasm, polar filament, polar cap, anterior and posterior vacuole, and spore shell were observed in TEM study. The polar filament ran oblique course backward from its base at the centre of the polar sac, narrowed slightly in diameter gradually upto the end and formed a coil in peripheral layer of cytoplasm. The number of coils was 10-12. Life cycle includes two distinct stages, spore and vegetative. *N. ricini* was found highly species specific.

Keywords: Eri silkworm, microscopy, *Nosema ricini*, pebrine disease, *Philosamia ricini*, polar filament

INTRODUCTION

Natural silk is produced in sixty countries. China (71%) and India (17%) are the major producers of silk. India has a unique distinction of being the only country in the world producing four varieties of silk viz. mulberry (89.6%), tasar (17.4%), eri (8.1%) and muga (0.6%). The uniqueness of North-Eastern (NE) states of India is the existence of all types of sericigenous insects (mulberry, tasar, eri and muga) in one place, belonging to Trans-Himalayan Zone. As a whole the climate of the NE states are very much conducive for abundance of insect fauna and their amazing biodiversity. This area has 64.4% forest cover and harbors rich endemism (Bhattacharya and Teotia, 1998). Assam, Meghalaya, Manipur and other North- Eastern states are the only states producing eri silks. About 90% of eri silk in India is produced in North-East region, mostly in Assam (85%). Assam is the original homeland of ericulture (Chandrasekhar *et al.*, 1989). Eri silkworms are polyphagous and are domesticated. *P. ricini* is well known for its delicious and nutritious pre pupa/pupa. Eri silk is non-reelable in nature and used mainly for production of carpets, blankets, knit wears, denims and ready-made garments. Eri silk having unique characteristics like thermal properties, extra softness and aristocratic finish and is the part of the culture

and tradition of NE people. It is a new silk product in the international market including Japan, Kenya and Egypt. In India Central Silk Board promotes eri-culture for women employment and income for rural people for its sturdiness and easy acclimatization in different agro-climatic conditions. About 2460 metric tonnes of eri silk was produced in 2009-2010. Besides, eri silkworm is now also reared in non-traditional areas of South India. About 7.76 lakh tribal people are engaged in this industry for this reason it is called poor men silk (Patil and Savanurmath, 1989).

Ahimsa silk or eri silkworm (*P. ricini* Boisd.) feeds primarily on castor *(Ricinus communis*), Kasserou (*Heteropanax fragrans* Seem.), Papita (*Carica papaya* L.). Some secondary hosts plants are Payam (*Evodia flaxinifolia*), Barkasserou (*Ailanthus* sp.), Gulancha (*Plumeria acutifolia*), Bhotera (*Jatropha curcus*), Korhe (*Sapium engenifolium*) are available in Assam (Sahay *et al.*, 1999).

All silkworms suffer from various diseases especially pebrine (Microsporidia: Nosematidae), Flacherie (bacteria), Grasserie (virus) and Muscardine (fungus). Several reports of crop loss are available due to all and specific diseases in silkworm in India which vary from 20 to 40% (Janakiraman, 1961; Hanumappa, 1968). About 36% crop loss is attributed due to pebrine disease with occasional crop failure (Nataraju *et al.*, 2005). This disease of silkworms seems to be the primal constraint in the production of raw silk. Thus these pathogens have a great role in deterioration of sericulture industry. In Assam, incidence of pebrine disease is very much common (around 11.2-17.9%). Mortality due to disease is more in eri silkworm and once infected continuous failure of crop occurs due to density dependent factor, as rearing is conducted in trays in colonial form. In present investigation morpho-metry of Eri pebrine spores under light and electron microscopy is reported, besides information on cross-infectivity and effect of pathogen on various economic parameters is described.

MATERIALS AND METHODS

Collection, isolation, purification, counting and storage of pathogens

N. ricini were collected from live infected pupa of *P. ricini* (Breed: white plain) supplied by Eri Silkworm Seed Project Centre, Mirza, Kamrup, Assam (India). Isolated pure spores were taken for morphological observations at different stages of life cycle. The pathogens were propagated in eri larvae and purified from moths using percoll cushions (Patil, 1993; Bhattacharya *et al.*, 1994). The technique involved collection of spores of *Nosema* sp. from pebrine infected moths by centrifugation in endotoxin free water (E.F. water). To dilute denseness of microsporidia spores, a series of washes and centrifugations were made to remove dissolved substances, although small particulates contaminants still persisted. Centrifugation process concentrated spores near the bottom of residue. Infected moths (20-30) in 0.5% potassium carbonate solution were crushed in a mortar and pestle for easy separation of spores from different tissues. The suspensions were filtered twice through cotton, washed 3 times in E.F. water and centrifuged at 5000 rpm for 10 min. The sediments were treated with 2% KOH to dissolve fat globules so as make observation easy. The spore suspensions were then layered over percoll cushions and centrifuged at 10,000 rpm for 30 min. in a RC5C high speed refrigerated centrifuge (Sorvall) with swing-out-rotor (SH-MT12) to obtain purified *Nosema* spores. Neubaur hemocytometer (German Fine Optik) fitted with Thoma-Zaiss counting slide was used to count spores under compound microscope (x 600) following Undeen (1997). Lethal concentration (LC) 50 and 90 were measured following the standard procedure and used in dose dependent mortality study**.** Cold highly purified spore suspensions of most species remained viable for long time in distill water in a refrigerator (5±3˚C).

Morphometry: Light microscopy

One drop of molten agar (1.5% w/v) was put on a grease free micro-glass slide and one small drop (5 µl) of fresh pure concentrated (1.5×10^8 spores ml^{-1}) mature spores were taken in the middle of a cover slip for light microscopic observations. The cover slips were then put inverted position above the molten agar on slides so as to spread the spores on agar solution uniformly for measurement under

compound microscope. One hundred fresh spores were measured by adjusting the ocular scale in microscope and stage micrometer (Aneja, 2003). Leitz Diaplan phase contrast microscope was used for the study.

Morphometry: Scanning electron microscopy (SEM)

Electron microscopy technique was used to study various developmental stages of microsporidian and finer structures of spores so as to facilitate the differentiation of closely related species on the basis of specific characters, particularly the number of coils of polar filament and angle of tilt of coils. Fresh pure concentrated (2.5×10^8 spore ml^{-1}) mature spores (1.5 ml) suspended in distilled water was centrifuged at 6000 rpm for 10 min. for SEM study and pellets containing mature spores allowed for primary fixation with 2.5% glutaraldehyde followed by post-fixation with 1% osmium tetraoxide (Undeen, 1997). Pellets were dehydrated through grades of ethyl alcohol with two changes in each grade. One drop of dehydrated sample was placed on upper surface of cover slip, dried and cover slip fixed on stud with cello-tape having both sides adhesive. Silver-based paint was used to ensure conduction between cover slip and metallic copper stud. The stud was then put into vacuum evaporator connected with high electric volt (5-20 KV) to put gold vapour coating at 200-300°A over the sample of stud. The metallic stud was put in respective place of SEM and required snaps of selected material at desired magnification taken. S-530 Hitachi (Japan) was used for SEM; HCP-2 (Japan) for critical point drying and IB2 Ion Coater, Eiko Engineering (Japan) for gold coating.

Morphometry: Transmission electron microscopy (TEM)

Pure and fresh concentrated mature 2.5 ml spores suspension (2.5×10^8 spore ml^{-1}) was allowed for primary fixation with 2.5% glutaraldehyde followed by post fixation with 2% osmium tetroxide hardened in 1.5% molten agar (w/v) as per standard technique for TEM study (Undeen, 1997; Sato *et al.,* 1982). Only golden and grey sections were stained with uranyl acetate and lead citrate. The sections were observed in e/m as uranyl acetate was used as en-block stain. The grid was placed on respective boxes of GEOL GEM 100 CX electron microscope and seen at 60 KV with x15000 magnification.

Life cycle

The 5th stage '0' h larvae were orally inoculated with microsporidian spore (1×10^6 spore ml^{-1}) to study the life cycle stages as per the procedure of Ishihara (1969). After 24 to 144 h oral inoculation, mid gut of infected larvae were collected at 24 h interval, dissected out and kept in 50% alcohol to clean the gut contents. The gut contents were smeared on slides as per Larson (1999) and allowed for fixation in absolute methanol followed by Giemsa staining. The slides were passed through upgraded ethanol for 10 min. at each step before passing the slides to absolute ethanol. The slides were hydrolyzed in 1N HCl at 60℃ for 8 min and observed under compound microscope in 1000X magnification. All gut tissues were fixed in 2.5% gluteraldehyde and 2% osmium tetroxide; and stained with uranyl acetate and lead citrate (Undeen, 1997). Leitz Diaplan phase contrast microscope (x 1000) and GEOL GEM 100 CX electron microscope seen at 60 KV with x15000 magnification was used for the study.

Cross-infectivity

N. ricini collected from eri silkworms were taken as pathogens as per Patil (1993). Ten disease-free layings of *P. ricini* were collected, 50 eggs were crushed in a mortar and pestle and tested as a sample under compound microscope to ascertain their freeness from any disease. Like-wise disease-free layings of mulberry (*Bombyx mori*), tasar (*Anthearaea mylitta*) and muga (*Antheraea assama*) were collected from different places and eggs tested. After hatching, 180 larvae from each type, mulberry (*Bombyx mori*), tasar (*Anthearaea mylitta*), muga (*Antheraea assama*) and eri silkworm, were divided into 3 replications of 60 larvae in each replication and maintained separately. Mulberry larvae were allowed to grow till 2nd moult and after resuming from moult 3rd instar '0' h (3rd instar 1st day) larvae were fed mulberry leaves (*Morus* sp. var. 'S_{1635}') smeared with *N. ricini* suspension containing 1.52×10^8 spores ml^{-1}. The 1st instar`0'h tasar, eri and muga larvae (1st day 1st instar) were fed fresh Arjuna (*Terminalia*

arjuna), castor (*Ricinus communis*) and saulau leaves (*Litsaea polyantha*), smeared with *N. ricini* suspension containing 1.52 x 10^8 spores ml^{-1}. The colonies of silkworm were reared at room temperature on fresh leaves *ad lib* under ambient conditions as per the standard rearing method. Leaf surfaces were sterilized before feeding silkworms. Short rinses in 75% ethanol followed by distill water rinse and air dry was used to eliminate the influence of pathogens in leaves (Sheeba-Rajakumari *et al*., 2007). Care was taken to ensure that all insects were healthy and robust at the time of experiment.

For inoculation leaf dishes (28.27 cm^2) were dipped in 1 ml pathogen spore suspension, semi-dried and then 6 larvae allowed to feed on one dish for 6 h. Ten dishes for 60 larvae in each replication and 30 dishes for 180 larvae in each batch were maintained. The leaves smeared in distill water were fed to another batch of 180 silkworm larvae as healthy control. Another batch of 180 silkworm larvae was fed leaves with pathogen (LC_{90}). *N. ricini* contained 1.52 x 10^8 spores ml^{-1} as infected control. Rearing (brushing to completion of spinning) was conducted. The Observations were recorded for a week and all dead larvae 4 day after inoculation were examined under microscope for confirmation of mortality due to pebrine infection.

Economic parameters

Dead larvae 4 day after inoculation with pathogen *N. bombycis*, *N. mylitta*, *N. assamensis* and *N. ricini* had 1.52 x 10^8 spores ml^{-1} examined under microscope for confirmation of mortality due to infection and mortality of larva were recorded. Before spinning, mature larval weights were taken. After the cocoon formation, healthy and infected cocoons were weighed to assess the effect on cocoons. Shell weights were also taken in the same way. Data was statistically analyzed using ANOVA.

RESULTS AND DISCUSSION

Morphometry: Light and electron microscopy

Mature spore were elliptical in shape, blunt or tapering at both ends measuring 3.78 ± 0.06 µm in length, 2.63 ± 0.03 µm in breadth and 13.69 μm^3 in volume. The spores had huge refractive index and appeared greenish in colour (Plate I a, b). Mature spore under SEM revealed slight concavity or tapering at both ends with smooth external morphology and had hard spore wall (Plate I c, d).

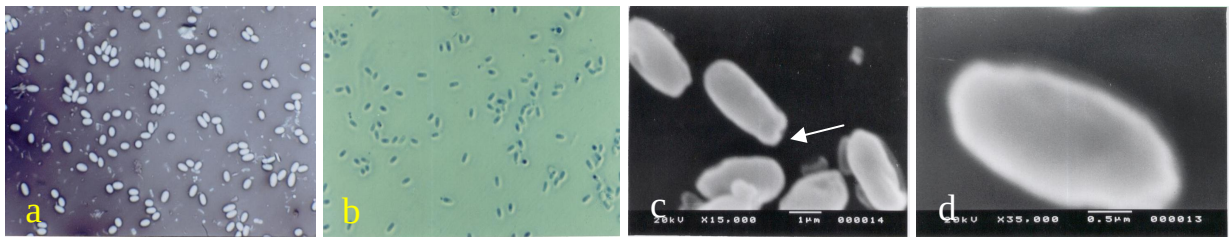

Plate I: a) *Nosema ricini* in Nigrosin (x1000); b) *N. ricini* in water suspension (x1000); c) *N. ricini* SEM: showing concavity or tapering by arrow (x15000); d) *N. ricini* in SEM (x35000)

TEM structure of microsporidian spores revealed that main components were sporoplasm, polar filament, polar cap, and posterior vacuole and spore shell (Plate II a). The sporoplasm was stretched in the form of a girdle across the width of spore and it contained a pair of nuclei. Polar capsule was projected into the cavity of spore; articulated with spore wall at the anterior end and connected to the exterior through a small opening. The polar filament was tubular, coiled, and spring-like structure lodged within polar capsule. Sporoplasm was high in electron density and had two nuclei surrounded by double-layered outer exospore and an inner endospore. Anterior part of spore was covered with a polar cap. The edge of polar cap extended along the inner wall of spore. The coiled part of polar filament was located along the inner wall of spore shell in a single row. A fine electron dense border was present at the inner edge of endospore. Exospore was uniformly thick, smooth and uniform coating. The

endospore was electron lucent and considerably thinner and attained 200 nm or less at the anterior end. A dome-shaped cavity was curved out at the inner side of endospore. Lining this cavity and extending backwards close to the spore wall was a membrane bound spore sac which resembled the cap of a mushroom. The central region, a somewhat expanded cavity, sealed the base of polar filament. The sac was separated from the wall by a narrow band of cytoplasm and by plasmalemma which extended around the entire cytoplasm complex in spore cavity.

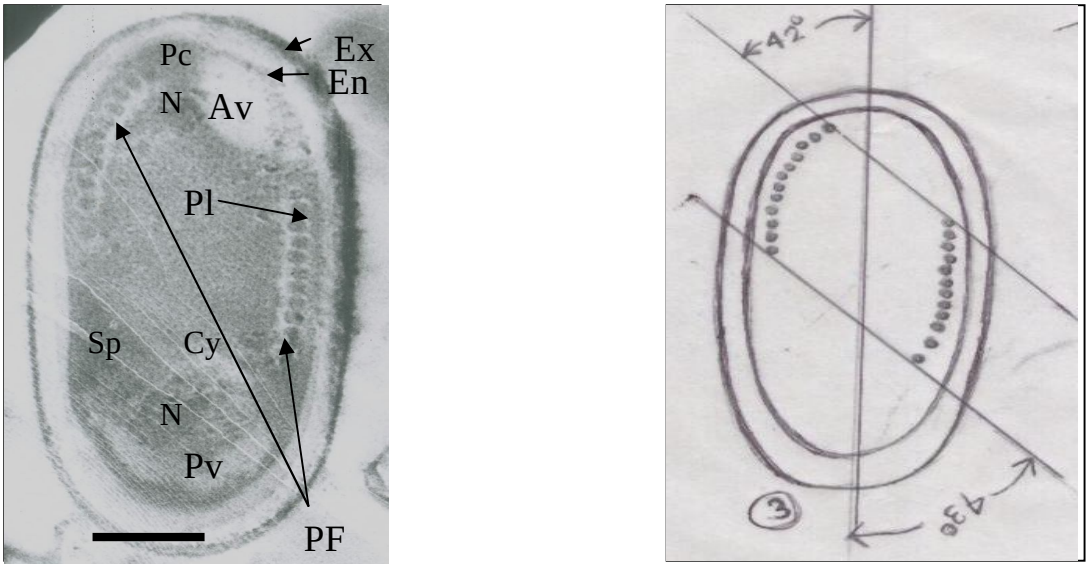

Plate II: Left side: *N. ricini,* enlarged TEM photographic view showing polar filament (PF), nucleus (N), exospore wall (Ex), endospore wall (En), anterior vacuole (Av), posterior vacuole (Pv), polar cap (Pc), sporoplasm (Sp), cytoplasm (Cy) and plasmalemma (Pl). Bar represents 1 µm; Right side: TEM photograph showing angle of tilt (degree) of a mature spore showing nucleus (N) and of *N. ricini* (3) angle indicates by arrow (x 15000).

This paper for the first time documents a detailed morphology and life cycle of *N. ricini* in light, scanning and transmission electron microscopy and therefore no other retrospective observation is available. Chakrabarty and Manna (2006) described the *N. ricini* found in eri silkworm, *P. ricini* (Breed: white plain) based on detail study on light and electron microscopy and life cycle. It was felt necessary to study minimum ultra-structure characters for the identification of microsporidian genera. In present study light microscopy, SEM and TEM of *N. ricini* showed differences in shape, size and measurement of spores compared to other microsporidian spores. The shape of spore is considered as a peculiar distinguishing character for genus *Nosema* and all the species of this genus share a basic spore shape (Larson, 1999). SEM and TEM showed better morphological and structural details of parasite (Santeeskumar and Ananthan, 2004). Present finding is the agreement with all ultra-structural features of the spores of genus *Nosema* documented so far (Sato *et al*., 1982; Canning and Vavra, 2000). The pansporoblastic membrane of *N. ricini* was not enclosing the spore in the present observation, a distinct character to the genus *Nosema* (Jouvenaz and Hazard, 1978).

The polar filament ran an oblique course backward from its base at the centre of polar sac, narrowed slightly in diameter gradually up to the end and formed a coil in the peripheral layers of cytoplasm. The number of coils in spore of *N. ricini* was 10-12. In anterior region of spore, the cytoplasm was organized as a series of membranous sheets surrounding the straight sections of polar filament. The precise organization of membranes varies in different species. Two nuclei occupied the centre of spore within the coil and were surrounded by cytoplasm and undifferentiated, except for the abundance of ribosomes and a few cisternae of endoplasmic reticulum. Posterior vacuole lied within the coil at the posterior end and contained sparse amorphous materials. In present investigation, only one

type of spore (monomorphic) of *N. ricini* was observed in light microscope, SEM and TEM. So, pebrine disease in eri silkworm, *P. ricini* is solely caused by the infection of *N. ricini* having 10-12 numbers of coils of polar filament. Monomorphic microsporidian, *Eurypancreaticum* having 10-12 numbers of coils of polar filament (Colley *et al.,* 1975) and *N. diphterostomi,* having 6-7 numbers of coils of polar filament (Leveron *et al.,* 2004) support the present findings. Monomorphic uninucleate environmental spore of *Pleistophora* sp. infect only midgut epithelium of silkworm (Kawarabata, 2003). However, in present study, monomorphic environmental spore of *N. ricini* infected midgut as well as fore and hindgut, silk gland, muscle, gonad, fat body tissue, etc. The majority of *Nosema* species has 11 coils; and out of 15 species reported so far (Milner, 1972a) *N. ricini* have 10 to 12 coils. However, neither the number of coils nor the arrangement within spore appears to be correlated with the genus *Nosema* (Milner, 1972b).

Coils of polar filament were arranged at a low angle to the long axis of spores which can be called as angle of tilt. The tilts measured from TEM pictures of spores were 42 and 43º from most anterior and most posterior coil (Plate II b). The angle of tilt discriminated the *N. ricini* clearly compare with other microsporidian which may be very much acceptable to identify it separately. The study of tilt was undertaken as typical number of coils in the polar filament and their angle of tilt to the long axis of spore distinguished the two closely related species (Burges *et al.* 1974). *N. oryzaephili* and *N. whitei,* are both similar in shape and size as seen by light microscopy. Surface of spore are smooth in SEM and internal structure of the spore under TEM are also similar, except in the number of coils (11 and 13) on the polar filament and angle of tilt (31º and 41º) which identify them into two new separate species (Burges *et.al.,* 1974). Recently Garcia and Becnel (1994) established 8 new species of microsporidia (Microspora) from *Argentina mosquiti* (Diptera: Culicidae) on the basis of number of coils in polar filament and angle of tilt. Both Kudo and Daniels (1963) and Sprague (1965) felt that internal structure of microsporidian spores differ much among various species. As more data from electron microscope studies accumulate, the similarities become more impressive than their dissimilarities. It appears that all species studied so far under electron microscopy have the same fundamental pattern of spore structure. The differences seem to be small or large variations of basic pattern (Sprague *et al*., 1968).

Life cycle

The life cycle of *N. ricini* included two distinct stages viz., spore and vegetative stage (Plate III, IV). In spore stage the cytoplasm occupied entire intra-sporal cavity under TEM. The membranous structure of polaroplast was given the appearance of anterior vacuole and closely wounded coil of the filament. The polar capsule was a sac-like structure that bulged out into the spore cavity from the anterior end. It was surrounded by sporoplasm and connected at the end to the outer membrane of spore and was communicated with the outside through a small opening. In vegetative stage, the spores entered the silkworm through feeding of contaminated leaf of food plants. After reaching the midgut, the polar filament was extruded. The two nuclei of spore divided into four and, at the same time the sporoplasm with two nuclei got injected into midgut tissue through evaginated polar filament. The outer two nuclei were degenerated inside the empty spore case. Subsequently, the polar filament was digested in alimentary tract and the sporoplasm with two nuclei was transformed into binucleate *amoebula* stage. The two nuclei of *amoebula* were united to form a uninucleate plannont**,** an extracellular stage. The plannont was 0.5-1.5 µm in length and formed within 48 h. It was spherical or globular in shape, devoid of cell wall and reproduced by binary fission. The plannonts were distributed and auto-infected in various tissues of host including gonads, Malpighian tubules, silk glands, etc. After reaching gut cell wall, it stopped movement and soon became covered by a membrane. It transformed into a sedentary form meront or schizont**.** Meront or schizont was intracellular, cylindrical or spherical or pear shaped and larger in size stage with a definite cell wall. It was formed within 2-3 days after inoculation. It reproduced by either by budding or by binary or multiple fissions. It elongated and developed into a sporont and finally into a spore. The host cell wall was then broken and spores liberated. This was the sporogony part of the life cycle.

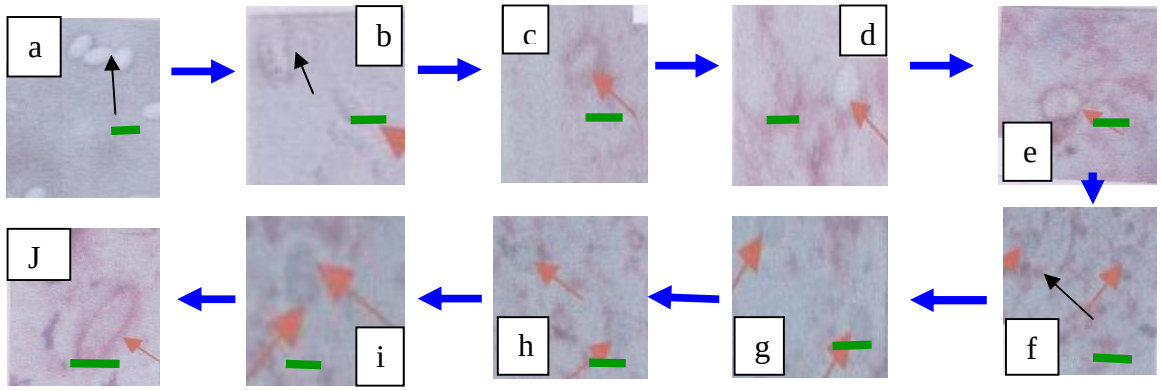

Plate III : Life cycle of *Nosema ricini* stained in Giemsa (1000X): a) Mature spore, b) Spore at release of polar filament, c) Spore after release of polar filament, d) Spore are completely everted polar filament, e) Uninucleated plannont, f-h) Various shape of binucleated schizont, i) Binucleated schizont showing 2 nuclei (N), & j) Maturing spore. [Spores indicated by arrows. Bar = 5 µm]

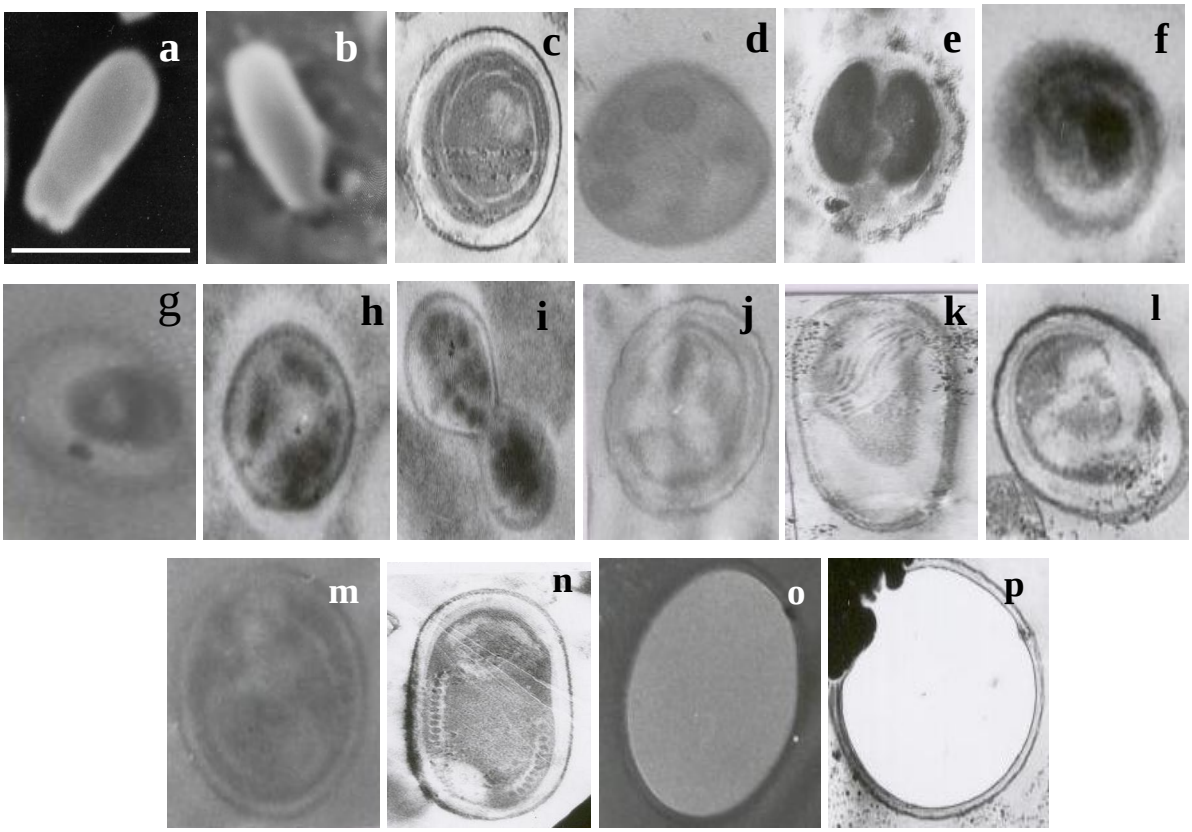

Plate IV: Life cycle of *Nosema ricini* (by TEM). Mature spore (a), Spore releasing polar filament (b), Sporoplasm with two nucleus(c), Sporoplasm with four nucleus(d), Fusion or division of two plannont (e), Uninucleated Schizont (f), Binucleated Schizont (g), Tetranucleated Schizont (h), Schizont with eight nucleus(i), Formation of polar filament(j), Formation of internal structure and coil of polar filament(k), Maturing spore(l-m), Mature spore (n) and Degeneration of spore case (o-p). Bar = 5µm for all pictures.

Table 1: Light, SEM and TEM structure of mature spore of *N. ricini*

Characters	Description
General	The spore of *Nosema ricini* is small in size
Spore shape and size	Elliptical in shape, blunt or tapering at both ends. It has huge refractive index and appears greenish in colour (Plate I a, b)
Length	3.8 μm ± 0.06 μm in length (Plate I a, b)
Breadth	2.6 μm ± 0.03μm in breadth (Plate I a, b)
Length/ Breadth	1.4 ± 0.02
Volume	13.7 μm^3
SEM study	Mature spore had slight concavity or tapering at both ends with smooth external morphology. It was provided with hard spore wall (Plate I c, d).
TEM study	The main components of the spores: sporoplasm, polar filament, polar cap, polar sac, nuclei, anterior and posterior vacuole, and spore shell recognized in TEM (Plate II a, b).
Sporoplasm	Sporoplasm was high in electron density with two nuclei surrounded by double-layered outer exospore and an inner endospore (Plate II a, b).
Polar cap	The anterior part of the spore was covered by polar cap. The edge of the polar cap extended along the inner wall of the spore (Plate II a, b)
Polar filament	The polar filament ran an oblique course backward from its base at the centre of the polar sac, narrowing slightly in diameter gradually upto the end and forms a coil in the peripheral layer of cytoplasm. The coiled part of the polar filament was usually located along the inner wall of the spore shell in a single row. The Central region, a somewhat expanded cavity, sealed the base of the polar filament (Plate II a, b).
Endospore	The endospore was electron lucent and considerably thinner, attaining 200 nm or less at the anterior end. A dome-shaped cavity is curved out at the inner side of endospore (Plate II a, b).
Exospore	Exospore was usually uniformly thick, smooth and uniform coat in *N.ricini*. The endospore is electron lucent and considerably thinner, attaining 200 nm or less at the anterior end (Plate II a, b).
Polar Cap	Lining the cavity of endospore and extending backwards close to the spore wall was a membrane bound spore sac, which resemble the cap of a mushroom (Plate II a, b).
Polar Sac	The sac was separated from the wall by a narrow band of cytoplasm and the plasmalemma, which extends around the entire cytoplasm complex in the spore cavity (Plate II a, b).
Coils of polar filament	The number of coils 10 -12 was found in the spore of *N.ricini* (Plate II a, b).
Cytoplasm	In the anterior region of the spore, the cytoplasm was organized as a series of membranous sheets surrounding the straight sections of the polar filament (Plate II a, b).
Nuclei	The two nuclei occupied the centre of the spore within the coil surrounded by cytoplasm, undifferentiated except for an abundance of ribosomes and few cysternae of endoplasmic reticulum (Plate II a, b).
Anterior vacuole	It lie at the anterior end (Plate II a, b)
Posterior vacuole	Posterior vacuole lie within the coil at the posterior end and contained sparse amorphous materials (Plate II a, b).
Angle of tilt	Coils of polar filament were arranged at a low angle to the long axis of the spores, called angle of tilt, measured in degree. The tilts were measured from TEM pictures of spores were 42° and 43 ° from most anterior and most posterior coil (Plate II a, b).

In the present investigation, extra-cellular binucleate sporoplasm, plannont was observed in TEM after 48 h of *N. ricini* inoculation. The intra-cellular uninucleate, binucleate, tetranucleate and octanucleate meront / schizont were observed, very prominent after 96 h inoculations. Lom and Vavra (1963) found that the germ is injected directly into the host cell by means of polar filament. Ishihara (1968) demonstrated that *N. bombycis* is responsible for spreading the protozoa from cell to cell. No such retrospective observation is available. The observation supports the view of Nordin and Maddox (1968) who observed intracellular small binucleate and uninucleate schizonts, which appeared 48 h

after feeding spores. However, Ishihara (1968) and Ananthalakshmi *et al*. (1995) reported schizonts, sporonts, sporoblasts and secondary infective forms in the life cycle stages of *N. bombycis*. Sporoblasts formed a number of spores and released after maturation as noticed in the present study. This supports the sporogonial development in *N. pyllotreate* (Yaman *et al*., 2005). In present study cell wall of sporont was found thick perhaps due to fusion of Golgi vesicles (Vavra, 1965). The origins of filament and its internal structures were formed by the fusion and condensation of endoplasmic reticulum (Lom and Corliss 1967). Thick spore wall are resistant to a variety of environmental hazards (Undeen, 1997). It was observed that all the life cycle stages of *N. ricini* viz., Plannont, Schizont, Meront and Sporont, were prominent and the figures of the life cycle stages were quite similar with those of *N. assamensis*, except that *N. ricini* took less time (5-7 days) compared to *N. assamensis* (6 - 8 days) perhaps due to its domesticated nature (Chakrabarti and Manna, 2009).

Cross- infectivity

The presence of more than one species of pathogen in same habitat and harbouring the disease by any one of them renders the possibility of cross-infection amongst them. *N. bombycis, N. mylitta* and *N. assamensis* are not cross-infected to eri silkworms, *P. ricini*. *N. ricini* is highly species specific. But *N. ricini* cross-infected to muga silkworm, *Antheraea assamensis*, though no significant differences between cross-infected and control was observed in cocoon weight, shell weight, shell ratio percentage, cocoon length, cocoon circumference, larval duration, mature larval weight and effective rearing rate (ERR). The cross-infectivity of *N.ricini* to muga silkworm has also been observed by Mistry (1979) who reported that the growth of treated replication was low and unequal; and the mortality was quite high during larval instars and able to produce 22.6% infection. *N. ricini* was not able to cross-infected to mulberry, *Bombyx mori* and tasar silkworm, *A. mylitta*, is supported by Griyaghey and Sengupta (1989). However, larvae were unequal, unhealthy, irregular in moulting, larval period was more in treated larvae than control, mortality was 3.3% during Aug-Oct, 1977 and 15.71% during April-May, 1977, when cross infection of *Nosema* sp. of muga to eri silkworm was under taken (Mistry, 1979). It was reported that neither *Nosema* sp. of *A. mylitta* can infect the larvae of *P. ricini* nor *Nosema* sp. of *P. ricini* infects *A. mylitta* (Griyaghey and Sengupta, 1989). Healthy and infected eggs, larvae, pupae and moths of eri silkworm are depicted in pictures (Plate V, a-i).

Economic parameters

Mortality in eri larvae infected with *N.bombycis, N. mylitta* and *N. assamensis* was not noticed even after 3rd successive inoculation with high inoculum load of pathogen (LC 90, 1.52 x 10^8 spore ml^{-1}). However, 95% larval mortality was recorded when *N. ricini* was inoculated to eri silkworm in brushing stage, 2nd stage and 4th stage. Further, 95% of eri larvae died due to other diseases like viral and bacterial after 3rd successive inoculation with LC90 at 4th stage of silkworm inoculated with *N. bombycis*. Larval death due to viral/bacterial diseases was 5% and 78% after 3rd successive inoculation at 4th stage of eri larvae infected with *N. mylitta* and *N. assamensis, respectively* (Fig. 1). *N. ricini* with high inoculum concentration (LC90, 1.52 x 10^8 spore ml^{-1}) multiplied maximum in eri silkworm. Eri silkworms are only host for *N. ricini*. Larval weight was drastically decreased when it was infected by *N. ricini*. The eri silkworm infected with *N. bombycis, N. mylitta* and *N. assamensis* attained mature larval weight as like control batches (Fig. 2).

The cocoon weight of control batches was higher than any other infected batches. Lowest cocoon weight was observed in eri silkworm batches infected with *N.ricini.* There was an increasing trend from the batches infected with *N.bombycis,N. mylitta* and *N.assamensis* (Fig. 3). The shell weight decreased drastically when eri silkworm was infected with *N. ricini*. Shell weight of eri silkworm cross-infected with *N. bombycis, N. mylitta* and *N. assamensis* decreased slightly as compared to healthy batches (Fig. 4).

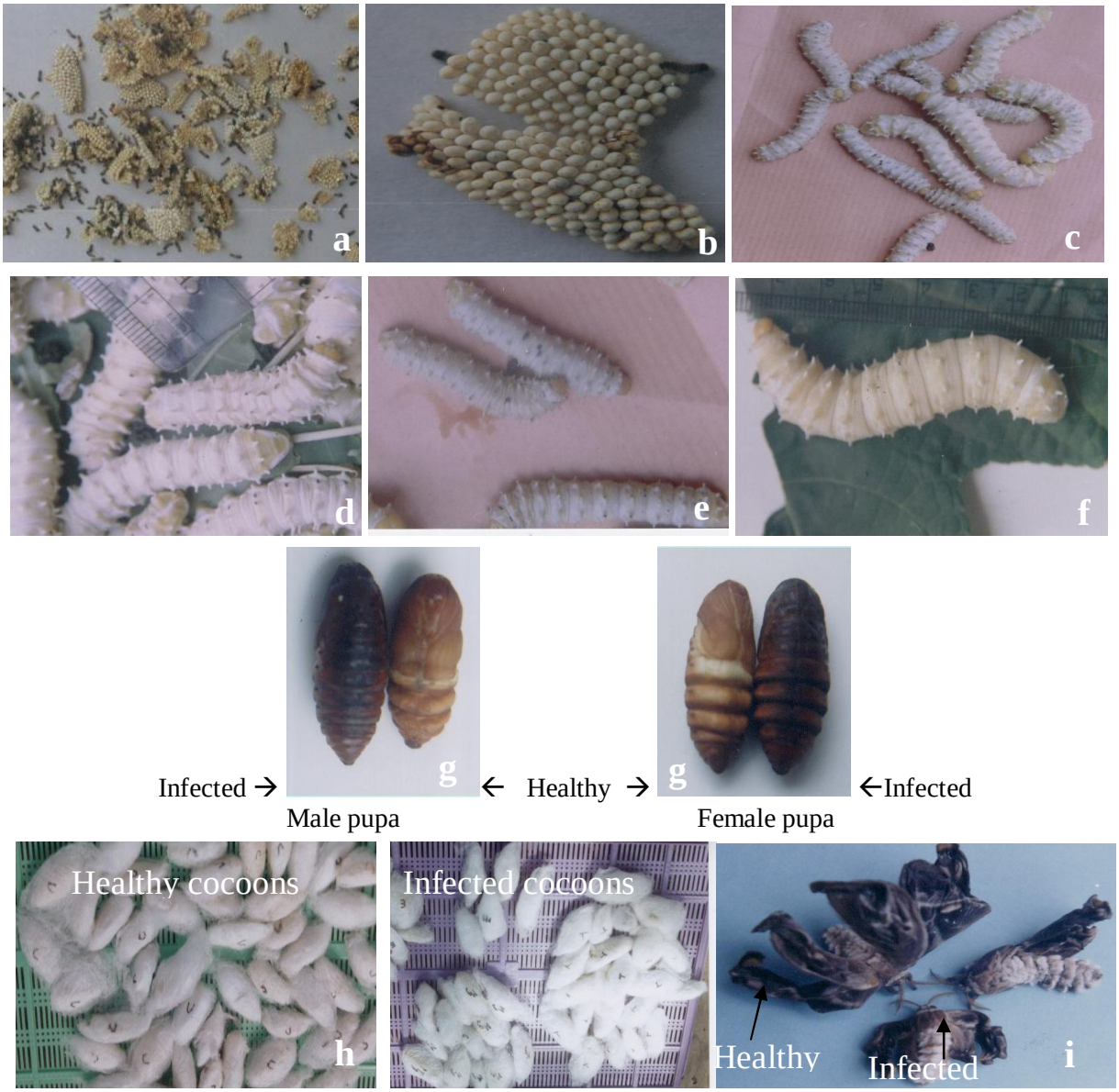

Plate V: a) Loose healthy eggs and hatched larvae of eri silkworm; b) Egg shell after hatching; c) Healthy larvae at 3rd stage; d) Healthy larvae at 4th stage; e) Pebrine infected 4th stage larvae; f) Healthy larvae at 5th stage g) Healthy and infected male and female pupae h) Healthy and infected cocoons i) Healthy and infected male moths

The number of spores (intensity of infection) that a host can harbour and still function normally is important in determining the role of a microsporidia as parasite in nature. The dose (LC90, 1.52 x 10^8 spore ml^{-1}) for *N. ricini* was found effective for high larval mortality (> 90%) as well as moths. Both temperature at which the host was maintained and the size of original inoculum (LC90) were found effective for high mortality. Higher temperature (32°C) and high spore dose (1.52 x 10^8 spore ml^{-1}) of *N. ricini* seem possible reasons for high mortality (> 90%) in eri silkworm during Aug-Sep. The minimum number of spores required to initiate a disease in silkworm was estimated which appeared to vary with the age of host. In present study a minimum dose (LC50) 1.52 x 10^6 spores. ml^{-1} of *N. ricini* was responsible for mortality to eri silkworm, when infected to '0' h 1st stage silkworm.

The number of spores (intensity of infection) that a host can harbour and still function normally is important in determining the role of a microsporidia as parasite in nature (Ghosh and Saha, 1992). A dose of 10^5 *Nosema carpocapse* spores fed to 4th instar larvae of *Cydia pomonella* resulted in 8 x 10^6 spores / adult, whereas feeding 10^8 spores gave 2.9 x 10^7 spores / adult (Malone and Wigley, 1981). Although the infection did not cause mortality, it reduced the fecundity of infected moths. Wendels *et al.*(1976) reported average males and females as 25.4 x 10^6 and 38.9 x 10^6 spores per moth, respectively, these levels being detrimental to the host. In case of stored pest, *Lophocalerses pusillus*, 3.15 x 10^8 spores per larva and 2.5 x10^8 spores per adult is highly effective and causes mortality of 84% larvae and 62% adult. In present study similar effective higher dose (1.52 x 10^8 spore ml^{-1}) for *N.ricini* for mortality of larvae and moths has been recorded.

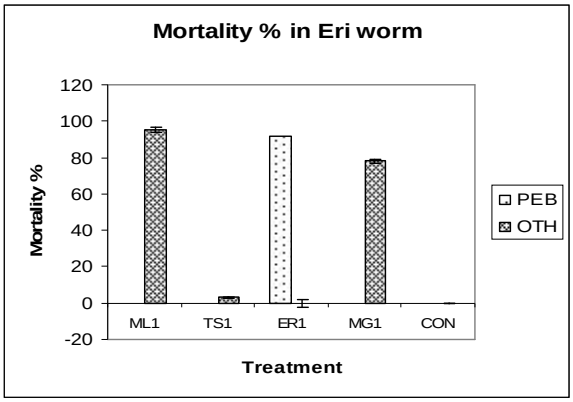

Fig. 1: Mortality % due to infection of *N. ricini* (ER-1) and other pathogens (ML1 – *N. bombycis*, TS1 - *N. mylitta*, MG1 - *N. assamensis*, CON - Control, OTH - Other pathogen). Bar represents the SE of square root of mean

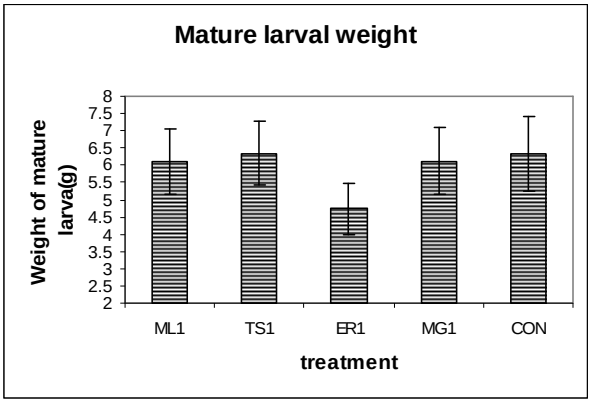

Fig. 2: Effect of pathogens in mature larval weight of *P. ricini.* (ER1 - *N. ricini*, ML1 - *N. bombycis*, TS1 - *N. mylitta*, MG1 - *N. assamensis*, CON - control). Bar represents the SE of square root of mean

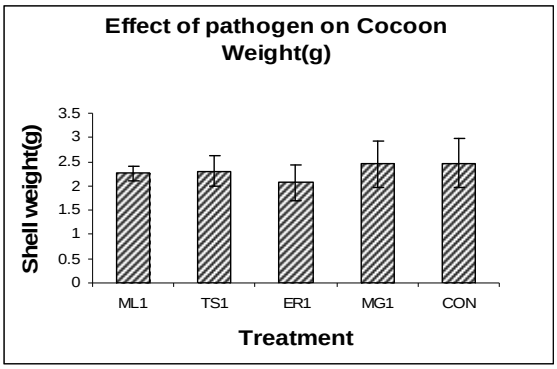

Fig. 3: Effect of pathogen in change of cocoon weight of *P. ricini*. (ER1 - *N. ricini*, ML1 - *N. bombycis*, TS1 - *N. mylitta*, MG1 - *N. assamensis*, CON - control). Bar represents SE of square root of means

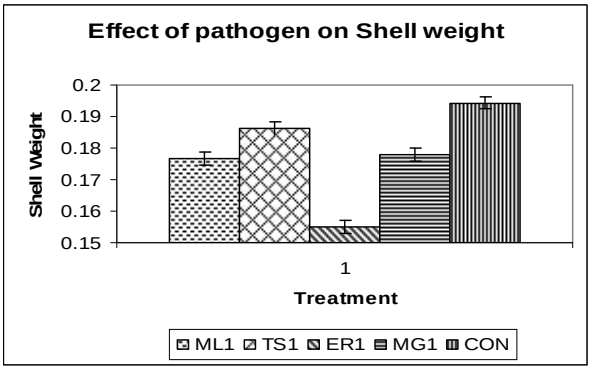

Fig. 4: Effect of pathogen in change of shell weight of *P. ricini*. (ER1 - *N. ricini*, ML1 - *N. bombycis*, TS1 - *N. mylitta*, MG1 - *N. assamensis*, CON - control). Bar represents SE of square root of means

Maddox (1968) reported in his quantitative study that generation time of *Nosema necatrix* varied with the spore dose administered to the caterpillar *Psedolatia unipuncta*. A small spore dose with subsequent low population density of vegetative forms resulted in delayed sporogony and, therefore, length ended the developmental cycle of the parasite. Both temperature at which the host was maintained and the size of original inoculum affected the generation time. As the temperature increased

to 32°C and spore inoculum increased to 10^6 spores per larva, the generation time decreased to a minimum 99 h. But when the temperature was decreased to 15°C and spore inoculum decreased to 10^4 spores per larva the generation time increased to more than 1000 h. In present study, higher temperature and higher spore dose (1.52×10^8 spore ml^{-1}) of *N. ricini* resulted in higher mortality in muga silkworm during August-September.

The maximum number of spores required to initiate a disease in silkworm has been estimated. It appears to vary with the age of host. It has been demonstrated that 1-10 spores are sufficient to cause disease in 2nd instar larvae, whereas 100 spores are required in 5th instar larvae to produce similar symptoms (Patil, 1993). The longevity of instars and the amount of feeding are some of the factors which influence disease manifestation (Ishihara and Fugiwara, 1965). In present study, a minimum dose of 1.52×10^6 *N. ricini* spore ml^{-1} caused mortality to eri silkworms when infected to '0' h 1st stage silkworm.

Interaction of different microsporidians with host or different hosts with one microsporidia involves as assessment of variations. Sometimes, the infection in one host is acute and chronic in another. Acute infection, as in case of *N. bombycis*, resulted in the death of host within 10-14 days, whereas chronic infection resulted in mortality during pupation or adult moth. In an apparent infection, spores and vegetative spores may be present but the rate of multiplication is slow that regeneration replaces all the infected cells and there is no visible alteration in host (Kishore *et al.,* 1994). This observation get supports from present findings, where infection of *N. ricini* to reduce larval span of muga silkworm, another host used in this experiment, as the cases are acute and increase the larval span in eri silkworm in chronic cases.

Eri silkworm was found maximum resistant than other silkworms. After repeated inoculation larvae became weak and easily infected by other diseases. Thangavalu (1989) stated that eri silkworm is comparatively more resistant to disease and tolerant of high fluctuation of temperature and humidity. Prasad and Saha (1992) have informed that eri silkworms are resistant to different diseases and can withstand extreme climatic conditions. *N.ricini* is able to produce pebrine disease in *P. ricini*, as this pathogen is highly species specific (Thangavelu, 1989).

Conclusion: *N. ricini* is highly species specific. Interaction of different microsporidians with single host or one single microsporidia with different hosts involve as assessment of variations. Sometimes, the infection in one host is acute but chronic in others. In present study infection of *N. ricini* was found effective for reduce larval span of muga silkworm as the cases were acute. It was maximum resistant than other silkworms and could withstand the extreme climatic conditions so eri culture has ample scope for expansion particularly in tribal belt of India. Besides, protein profiling need to be explored for isolation and identification of climate tolerant and disease resistant protein(s) from eri silkworm to make improved transgenic breed to control the silkworm diseases in other silkworm also.

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