STUDIES ON THE WHITE MUSCARDINE DISEASE OF THE SILKWORM, *BOMBYX MORI* LINNAEUS

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CERTIFICATE

This is to certify that the thesis entitled "STUDIES ON THE WHITE MUSCARDINE DISEASE OF THE SILKWORM, BOMBYX MORI LINNAEUS" submitted in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE (AGRICULTURE) in AGRICULTURAL ENTOMOLOGY of the University of Agricultural Sciences, Bangalore is a bona fide record of research work done by Mr. M.R. VENKATARAMANA REDDY during the period of his study in this University under my guidance and supervision, and the thesis has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles.

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INTRODUCTION

I. INTRODUCTION

Karnataka is the major silk producing State contributing more than 80 per cent of the Indian silk. Presently silk industry in India ranks fifth in the world and employs about 3.5 million people. It could be expanded all over India to exploit its potential to employ upto 10 million people. However, the silk yields per 100 layings are comparatively less both quantitatively and qualitatively mainly due to the diseases of silkworm. On account of diseases, rearing of silkworms for cocoons remained as the most uncertain of all the agricultural profession to the farmer (Mukerji, 1912). To obviate this uncertainity leading to severe loss in rearings and to enhance the prospectous of sericulture, the need for studying the silkworm diseases and their control is keenly felt since the begining of this century in India.

The infectious diseases in insects are caused by pathogenic micro-organisms like fungi, protozoa, bacteria, viruses and rickettsia. The occurence of maladies in insects was first noticed among domestic insects like silkworm and honeybee. One of the earliest scientific reports was made by Bassi (1835) on the fungus, Beauveria bassiana (Balsamo) - Vuillemin as the causal agent of the white muscardine disease of the mulberry silkworm, Bombyx mori Linnaeus.

The incidence of diseases on mulberry silkworm is known to cause a loss of 30 to 40 per cent in the silk yield in India (Janakiraman, 1961), over 30 per cent in developing countries of south-east Asia and 10 per cent in advanced countries (Nanavaty, 1965) like Italy, Japan (Yokoyama, 1963) and China (Kellog, 1928). The major diseases prevalent in different sericultural tracts of India are white muscardine, pebrine, grasserie, gattine and flacherie (Dasgupta, 1950).

Muscardine diseases, the most contagious and dreaded of all silkworm diseases, are of different kinds known by their colour (Mukerji, 1912) and are caused by about 20 species of entomopathogenic fungi (Steinhaus, 1949; Krishnaswami et al., 1973). The major one among them is the white muscardine disease, which caused a loss varying from 5 to 35 per cent in northern Italy (Anonymous, 1928) and upto 100 per cent in Karnataka State, India (Anonymous, 1975). The incidence of this disease has reached an alarming proportion, especially in the recent years.

Though many workers (Mukerji, 1912; Paillot, 1930; Steinhaus, 1949; Dasgupta, 1950) have contributed to the understanding of this disease, much remains to be done on its nature and symptomatology, the life cycle of the causative fungus, degree of susceptibility of different silkworm stages, fungal inoculum required for 50 per cent larval mortality and presence of resistance in different races of silkworm against this disease. The present investigation are in furtherance of such objectives to understand and combat this disease for better management of sericulture.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

White muscardine disease of silkworm, Bombyx mori L. perhaps was the first recognised disease and occurs through out the world where ever silkworm is reared (Steinhaus, 1949). It has been particularly disastrous in Italy, France (Steinhaus, 1949), Japan (Cartweight, 1933) and India (Mukerji, 1912; Dasgupta, 1950). It was designated first as 'mal del segno' (the disease having a sign) and now as 'calcino' to refer to the white powder like calcium of the white muscardined silkworm after it transforms into a mummy covered with white efforescence, in Italy. It is known in India by several vernacular names like 'sunnakaddi' or 'sunnakattu roga' in Karnataka (Janakiraman, 1961) 'Chunakete' or 'chitt' in West Bengal (Mukerji, 1912). However, it is known commonly all over the world as 'White muscardine'.

The term 'muscardine' has been commonly used to refer any type of fungal infection of insects instead of the precise term 'mycosis'. It was apparently originated from two words, 'muscardin' to refer dead, white powdery, mummified silkworm body as they resemble comfit or bonbon and 'muscadin' means a musk lozenge in addition to dormous ('muscardinus') condition. These words might have been derived from an Italian word, 'muscardino' means musk comfit, grape, pear and the like or any plant with musk scented foliage, flowers etc. to refer the white efforescence of the diseased worm.

The word 'muscardine' was used first to refer to the white muscardine disease of silkworm caused by Beauveria bassiana (Balsamo) Vuillemin and later to refer to a mycosis caused by another entomogenous fungus, Metarrhizium anisopliae-(Metchinkoff) Sorokin. As there are nearly 1000 species of entomopathogenic fungi (Chitra et al., 1975), 'muscardine' might be permissible to use in connection with those mycoses in which the fruiting bodies or conidia arise on the exterior of the insect corpse (Steinhaus, 1949).

White muscardine disease of silkworm was first reported in 1763 by Boissier, who stated that it was the resultant of particular atmosphere, called 'touffe' means 'wisps of heat', which preceeds storms. Nysten (1808) attributed it to the defective incubation of silkworm eggs. He mentioned negligence in rearing and cleaning, want of proper aeration and distribution of mulberry leaves, overcrowding of silkworms and state of prevailing atmosphere on the eve of the storm as other causes. Dandolo (1814) emphatically asserted that muscardine resulted from abnormal physiological conditions of the silkworm and reported that the white efforescence on silkworm cadavers was the 'original mineral'. Many other workers postulated the causes other than microbic influences. However, in 1834, Bassi (1835) discovered the fungus as the cause of this disease, showed its infectivity, parasitic origin, highly contagious nature and its multiplication in and on the body of the silkworm, for which he has been credited as the first investigator to discover the real cause of a disease (Fildes, 1951).

Balsamo (1835) studied and described the fungus and named it as Botrytis bassiana, the specific epithet in honour of Bassi. Audoin (1837a, b) supported and elaborated Bassi's discovery by artificial reproduction of the disease with injection of the fungus into the haemocoel or fatty tissue of a healthy silkworm, irrespective of larval instar or its age. He also showed, as stated by Bassi, the indispensibility of certain humidity for complete development of the fungus.

Contagious nature of this disease was doubted by Guērin-Menēville (1848). He believed that disease manifestations were merely symptoms of rearing in bad conditions with unskilled hands. He also reported that by proper handling of worms in more hygienic conditions, the silkworm resisted the invasion of the fungus. He found oval granules in association with blood globules while studying the blood of diseased silkworms and named them as 'haematozoides', which gave birth to mycelium and produced muscardine. These were later proved to be pebrine spores (Pasteur, 1870). However, Vittadini (1853) refuted the observations of Guērin-Menēville by conducting new experiments and found the fungus as the only and real cause of the disease.

Systematic position of the fungus

The genus Beauveria is one of the most well known fungi among pathologists because of its economic importance and

Bassi's masterly research on the fungus remained as a base for epoch making discoveries of Pasteur. It has been cited in many of the text books on pathology as the first instance in which a disease of an organism (silkworm), was proved to be due to a definite micro-organism.

As more and more species of *Beauveria* were reported their identity became more confusing and vague. Balsamo (1835) studied *B. bassiana* and placed it under genus *Botrytis* as *Botrytis bassiana* of which *B. cineria* Pers., a vine disease causing fungus as the type species. Beauverie (1911, 1914) studied *B. bassiana* in comparison with yet another closely related species, *B. effusa* Beauverie found on silkworms and showed some similarities between them. Thus he suggested to erect a separate genus for them. Vuillemin (1912) erected a new genus, *Beauveria* honouring Beauverie, with *Beauveria bassiana* (Balsamo) Vuillemin as the type.

Dieuziede (1925), Arnaud (1927), Lefebvre (1931) and a few others made comparative studies on *Beauveria* spp. including *B. effusa* (Beauv.) Vuill., *B. densa* (Link) Picard (= *B. tenella* Delacroix) and *B. globulifera* (Spegazzini)-Picard infecting the silkworms (Steinhaus, 1949). The genus *Beauveria* has been generally recognised and retained by most authorities, but Clements and Shear (1931) have placed this in synonomy with the genus *Phymatotrichum*.

Siemaszko (1937) concluded that out of ten species of *Beauveria* reported only three were tenable, namely *B. bassiana*

B. globulifera and *B. densa*, due to their differentiality from one another in pure culture. MacLeod (1954a) studied the genus *Beauveria* thoroughly and concluded that out of 14 species only two, *Beauveria bassiana* and *B. tenella* - (Delacroix) Siemaszko were valid. The farmer was characterised by globose and oval spores occuring in about equal proportion, consisting of the old species *B. acridiorum* Brongniart and Delacroix, *B. doryphorae* Poisson and Patay, *B. delacroixii* - (Saccardo) Petch, *B. effusa* (Beauv.) Vuill., *B. globulifera*, *B. laxa* Petch, *B. stephanoderis* (Bally) Petch and *Isaria vexans* Pettit as its strains.

According to the present usage, the taxonomic position of the white muscardine fungus is as follows:

Class : Deuteromycetes (*Fungi Imperfecti*)
Order : Moniliales
Family : Moniliaceae
Tribe : Verticillini
Genus : *Beauveria*
Species : *bassiana* (Balsamo) Vuillemin.

White muscardine fungus infecting other insects

The white muscardine fungus infecting insects other than silkworms was first recorded by Audoin (1837a, b). This is known to attack more than 150 species of insects belonging to several orders (Masera, 1936; Charles, 1941; Dresner, 1949; MacLeod, 1954 a,b; Aoki, 1971; Leatherdale, 1973; Bell, 1974).

Culture of the fungus

The fungus was found to be one of the most promising fungal pathogens in microbial control of insect pests. It has been studied in detail for its culture and nutrition.

The live host insects were used to culture the fungus in the past (Snow, 1890). Forbes (1895) attempted to grow it on non-living medium. Pettit (1895) tried artificial media to culture various entomopathogenic fungi. However, Johanys (1839) was the first to successfully cultivate this fungus on artificial medium of dead organic matter. Two principal types of media to-date used are those based on agar (notably Sabouraud's maltose agar and malt agar) and those modified substrates from plant material (Samsinakova, 1966).

The fungus B. bassiana has been cultured in vitro by various workers. Lefebvre (1931) used potato dextrose agar (PDA) and nine other dehydrated difco media. Dresner (1949) used molasses agar, lima bean agar in addition to PDA. Benham and Miranda (1953) used Czapek agar, corn meal agar, casein hydrolysate medium and Sabouraud's dextrose agar (SDA). SDA with two per cent yeast was used by Bao and Yendol (1971) and SDA with one per cent yeast and 0.5 g each of pencillin and streptomycin per litre medium was used by Wasti and Hartman (1975). MacLeod (1954a) used various media like

Sabouraud's maltose agar, molish medium, blood agar base, Raulin - Thom and Czepek - Dox media and corn meal agar. The important substrates from plant material, most frequently used are corn meal (Forbes, 1895) and bran (Dresner, 1949; York, 1958; McCoy and Carver, 1941). Martignoni (1964) has reviewed the suitable substrates used for mass production of different insect pathogens.

Schaerffenberg (1964), El Basyouni et al. (1966), Samsinakova (1966) and Aoki and Chigusa (1968, 1970) have studied various nutritional re-quirements of B. bassiana in detail and also their metabolism (Barnes et al., 1975).

Symptoms of the white muscardine disease of silkworm, B. mori.

The attack of insects by entomogenous fungi results in the production of a number of signs and symptoms, characteristic of particular host - pathogen interaction along with the combination of the environment (Madelin, 1963). The external and behavioural symptoms, have been recorded by various workers, at different stages of the silkworm.

Egg:

There were no external symptoms observed in dead muscardined eggs though spores of the fungus on the surface of the eggs were detected. Madelin (1963) stated that the muscardined eggs of insects changed their colour in general.

Larva:

In early stages of white muscardine disease of the silkworm there appeared no external symptoms (Tanaka, 1964). Careful observations revealed the sluggishness of the worms and increased rate of dorsal heart beat (Anonymous, 1956; Janakiraman, 1961). As the disease progressed larvae became inactive, pale in colour and lost its appetite (Mukerji, 1912; Tanaka, 1964). The elasticity of the larval body decreased (Mukerji, 1912; Anonymous, 1956; Krishnaswami *et al.*, 1973) and the areas surrounding spiracles and or intersegmental parts turned into dark brown colour. Later these areas became black spots on the larval body (Kurisu, 1962), due to the result of reaction of tyrosinase complex on tyrosine and or its intermediaries in the presence of oxygen. These specks oozing oily substance was observed by Kurisu (1962) and Krishnaswami *et al.* (1973). The larvae became more inactive and ceased movements (Anonymous, 1956) with frequent vomits and diarrhoea with agony (Tanaka, 1964; Krishnaswami *et al.*, 1973). Krishnaswami *et al.* (1973) have reported that the larvae were found to stretch their bodies upon the litter and became soft to touch. Larval mortality occured in 2 to 10 days (Arbousset, 1893; Mukerji, 1912; Krishnaswami *et al.*, 1973; Chitra *et al.*, 1975). The larval corpse turned pink or reddish in colour due to the secondary invasion of antagonistic red pigmented bacterium, *Serratia marcescens* Bizio (Masera, 1936; Steinhaus, 1949). This occurred in a few hours

after death of the larvae(Balsamo, 1835; Mukerji, 1912; Masera, 1936; Steinhaus, 1949; Tanaka, 1964; Krishnaswami et al., 1973). The white efforescence started initially on the intersegmental regions and in a day or two after death it covered the whole larvae (Steinhaus, 1949; Krishnaswami et al., 1973). Ultimately the corpse dried and transformed into mummies resembling 'lime covered sticks' or'bird's excreta'. The presence of white crystals on the body surface of the cadavers was reported to be the double oxalate of magnesium and Ammonia (C_2O_2Mg, 5 C_2O_4 $(NH_4)_2$, $10H_2O$) by Verson (Steinhaus, 1949; Dasgupta, 1950). However its true nature and identity have not been confirmed.

Pupa:

Arbousset (1893) found the infected cocoons somewhat brighter in colour, transparently shaded and lighter in weight as compared to normal cocoons. The hardening of muscardined pupae and white efforescence over the body was also noticed (Arbousset, 1893). Krishnaswami et al. (1973) have reported that the weight of muscardined cocoons decreased to one third of its normal weight. They also reported that the cocoons with muscardined pupae inside sounded like dried cocoons, when shaken. The moths failed to emerge from infected cocoons (Arbousset, 1893; Krishnaswami et al., 1973).

Moth:

Infected moths were sluggish and lost interest in copulation (Arbousset, 1893). The hardening and drying of dead muscardined moths (Krishnaswami et al., 1973) and white efforescence over the body mainly on abdominal segments (Arbousset, 1893) have been reported.

Route of infection

The routes of infection were commonly the integument, the digestive tract, the tracheae and wounds (Broome et al., 1976). Vittadini (1852) reported that the fungus penetrated into silkworm body through digestive tube, integument and respiratory ways but with no scientific proof for the latter two ways. Maestri (1856) concluded that infection by way of digestive tube was conditioned by the 'state of the tube'. The infection in silkworms by the fungus, B. bassiana, Per Os was reported by Gabriel (1959) and also through spiracles (Anonymous, 1956).

Arnaud (1927) was partially successful to demonstrate the penetration of the fungus through epidermis. Paillot (1930) showed that the germ tube of the fungus spore penetrated the chitinous layer easily by dissolving with its enzymatic secretions. The enzymes concerned with process of dissolving the integument were mainly chitinolytic though proteolytic enzymes were also very important (Robinson, 1966). The enzymes chitinase, cellulase, protease and lipase in the germ tubes and mycelium were detected and quantitatively determined (Leopold and Samsinakova, 1970). Samsinakova et al. (1971) demonstrated the essentiality of each of these enzymes in penetration of the cuticle of greater wax moth, Galleria mellonella L..

The enzymatic activity of the fungus including germinability of spores was related to its pathogenecity to the silkworm (Baladacci, 1939). Robinson (1966) has reported that the germ tube of the conidia penetrated the cuticle atleast the epicuticle by mechanical pressure. He further concluded that endocuticle was penetrated by the germ tube by its enzymatic action, as the hyphae was found to be enlarged as compared to the thickness of the hyphae along epicuticle. Many workers (Steinhaus, 1949; Madelin, 1963, 1966; Bell, 1974; McCoy, 1974) inclined to ascribe the fungal entry into the insect through integument.

Life cycle of the fungus

The life cycle of the fungus, B. bassiana in the larval body of the silkworm, B. mori has been reported by Vittadini (1853), Paillot (1930) and Steinhaus (1949) is presented below.

The fungus found penetrating all over abdominal, cephalic and thoracic regions of larval body by germ tube of its conidia and grew with great rapidity. First it proliferated in the hypodermal region by producing branched or free mycelia in addition to cylindrical conidia and then it entered the haemocoel and drew nourishment from plasma and also the blood cells of the haemolymph. It has been found to ramify althrough inside the body of the silkworm, at the expense of fat bodies. A good account of this characteristic development has been reported by Vittadini (1853). The hyphal bodies and conidia circulated throughout the body. The circulation finally stopped as the blood became pasty. The fungus confined mainly to haemolymph till the larvae approached death. Just before or after death the fungus penetrated the adipose tissues, nervous system, malpighian tubules, silkglands, muscles and finally the gut. About 48 to 60 hr after death these hyphae and newly germinated conidia grew back out through the integument. This penetration took first on the weaker areas of the integument like, articulating intersegmental membranes, the pores of sense organs etc. and later all over the integument. These penetrated hyphal tips later soon borne conidia.

Some times B. bassiana produced coremia (Petch, 1931) and perithecia along with conidiospores on surface of the cadaver, but coremial production depended upon moisture conditions (Manteifel and Shaposhnikoff, 1927). It has been reported to form sclerotia in the larval body as a post-morteum phenomenon (Paillot, 1929). It is known to develop effectively at 80 per cent relative humidity though it invaded all tissues of corn borer larvae, Pyrausta nubilalis at relative humidities as low as 40 per cent (Metalnikov and Toumanoff, 1928) and this successfully infected Laspeyresia pomonella (L.) under low humidity (Ferreira, 1943).

The life cycle of the fungus in silkworm, B. mori varied but normally developed from spore to spore in 10 days in cold weather and in about 4 days in the hot weather in India (Anonymous, 1956; Janakiraman, 1961). This has been well studied on artificial media by various workers. The period between inoculation on the culture medium and abundant sporulation of the fungus took less than 7 days (Lefebvre, 1931; Steinhaus, 1949; Samsinakova and Misikova, 1973; Rao, 1975). The sporulation was found to start on 6th day of inoculation in submerged culture (Samsinakova, 1966).

Susceptibility of different stages of silkworm

Except egg all other stages of silkworm have been reported to be susceptible to the disease (Steinhaus, 1949). Though all the instars of larvae were susceptible (Arbousset, 1893; Steinhaus, 1949; Dasgupta, 1950), the third and subsequent instars were more susceptible (Arbousset, 1893). Tanaka (1964), Masera (1937) and Dasgupta (1950) have reported that V instar larvae were more susceptible than other instars. Larvae in moulting reported to be most susceptible compared to the instars (Mukerji, 1912), as the larvae in moulting were inactive. Most of the infected larvae died of the disease caused by Aspergillus flavus, but occassionally the larvae showing mild black spots survived as these spots dissappeared after moulting and the larvae continued to live free from the disease (Kawakami and Mikuni, 1973; Kawakami, 1975).

Cocoons were also susceptible to the fungus (Arbousset, 1893), but of very rare occurence in nature. Pupae of the silkworm was also reported to be susceptible, but comparatively less susceptible than larvae, due to its cuticular antifungal agents such as saturated fatty acids, namely capric acid and caprylic acid (Koidsumi, 1957). Sussman (1951, 1952) has reported the susceptibility of papae of Cecropia silkworm to A. flavus infection. He further reported that the older pupae were less susceptible than newly formed pupae.

Moths were not only susceptible to the disease but also found to succumb more rapidly to the fungus than the larvae (Arbousset, 1893; Krishnaswami et al., 1973). On the contrary, Mukerji (1912) reported that moths were never infected by B. bassiana in nature. Gravid moths were more susceptible than the ones already laid eggs (Arbousset, 1893).

Susceptibility in different races of silkworm

Aquired immunity in silkworms to the fungus, B.bassiana (Paillot, 1930) and to M. anisopliae (Glaser, 1925) has been reported. The humeral immunity in larvae of various Lepidopteran insects has been reported (Briggs, 1959). Individual silkworms showed very little immunity against the infection by B. bassiana, according to Steinhaus (1949). However, Dasgupta (1950) reported that the silkworms showed no natural immunity against this disease. Koidsumi (1957) demonstrated the presence of immunity in silkworm due to its cuticular lipids.

It is well known that several races of *B. mori* differ in their susceptibility to the fungi, *B. bassiana* and *A. flavus* (Kawakami, 1975). The loss due to different diseases of silkworm including white muscardine disease was maximum in bivoltine races followed by the cross breeds and minimum in multivoltine races (Anonymous, 1975). A preliminary trial conducted in India showed that the different multivoltine races of the silkworm showed different degrees of the susceptibility (Anonymous, 1976).

MATERIAL AND METHODS

III. MATERIAL AND METHODS

Investigations on several aspects of the white muscardine disease of silkworm, Bombyx mori L. caused by the fungus, Beauveria bassiana (Balsamo) Vuillemin were carried out, during 1975-77, at the Department of Entomology, University of Agricultural Sciences, Bangalore.

Two silkworm rearing rooms at 200 m apart, one for rearing healthy larvae and the other for rearing diseased larvae, having similar conditions were selected. At least six days prior to the hatching of the eggs in each rearing season, the rearing rooms and equipments were cleaned, washed and properly disinfected with four per cent formaldehyde solution at the rate of about 800 ml per 10 Sq.m (Krishnaswami et al., 1973) using a compressed air sprayer. After this, the rooms were kept closed for 2 days for the effective formaldehyde gas diffusion. The samples of the washings of rearing rooms and equipments were plated on the culture medium of the fungus to observe the germination of fungal spores, if any, to make sure that the rooms were properly disinfected.

Silkworm Rearing

The hybrid Pure Mysore X Kalimpong A, a multivoltine race extensively grown in the State, was used for all the investigations including the screening of different silkworm races against the white muscardine disease. The eggs purchased from the Government grainages were surface sterlised with two per cent formalin solution, then washed with sterile water and dried in shade. Eggs were incubated at 25-27°C temperature and 85-90 per cent relative humidity (RH) over moist aand bed covered with perforated tissue paper. Eggs were kept in a dark place for 24 to 30 hr and exposed to diffused light when turned blue, to obtain uniform hatching (Krishnaswami et al., 1973). After 9 to 11 days of incubation all the eggs hatched before 11 A.M. Two hours after hatching, fresh tender mulberry leaves cut to about 5 Sq.mm were spread over the larvae on the egg cards. After all the larvae crawled on to the leaves they were 'brushed' on to a wooden tray with a paraffin paper at its floor and wet foam rubber strips all along its inner edges. Another paraffin sheet was used to cover the 'bed'. The paraffin sheets and wet foam rubber strips were used only upto the end of III larval instar except during the moulting periods to maintain the required RH. The trays were kept on a rearing stand with their legs resting in the 'ant wells'. The larvae were fed five times a day (Narayanan and Chowla, 1965) at 6 A.M., 10 A.M., 2 P.M., 6 P.M. and 10 P.M. with tender leaves cut to the required size upto III instar and whole coarse leaves to IV and V instar larvae. Kanva-2 variety of mulberry was used as food throughout the rearings. The 'Ripe larvae' were mounted on bamboo mountages and cocoons were harvested five days after spinning. Daily temperature and RH in the rearing room were recorded with the help of maximum and minimum thermometer and hygrometer throughout the study.

Isolation and culture of the fungus

The fungus was isolated by asceptically picking the conidia and mycelia from diseased silkworm cadaver and streaked on the poured plates of Sabouraud's maltose agar with one per cent yeast and 0.05 per cent each of Pencillin and Streptomycin sulphate to prevent bacterial growth. The fungal isolates, from muscardined silkworms collected from different localities in Bangalore, Kolar, Mandya and Mysore Districts of Karnataka State, were named as Isolates B, K, M and My, respectively. The Isolate B was used for further studies as it yielded larger quantities of spores and proved to be more virulent.

The fungus was cultured on seven media including Sabouraud's maltose agar with one per cent yeast, Sabouraud's dextrose agar with one per cent yeast, potato dextrose agar, yeast extract agar, nutrient agar, molasses agar and malt extract agar. The composition of these media is given in Table I and prepared according to the reference cited therein, maintaining the pH between 6.5 and 7.0. Inoculated media in petridishes or conical flasks were incubated at 25 to 27°C temperature and 95 per cent RH, as the fungus requires RH more than 92.5 per cent and temperature between 25 to 35°C for good germination, growth and sporulation (Walstad *et al.*, 1970). Sabouraud's maltose agar was subsequently used as it yielded more conidia.

Table I. Composition of the media for culturing the fungus, <u>Beauveria</u> <u>bassiana</u> (Balsamo) Vuillemin.

Sl. No.	Medium	Ingredient	Quantity (g/l dist. water)
1.	Sabouraud's maltose agar with one per cent yeast (Thomas, 1974)	Maltose	40
		Peptone	10
		Yeast extract	10
		Agar	15
2.	Sabouraud's dextrose agar with one per cent yeast (Bao and Yendol, 1971)	Dextrose	40
		Peptone	10
		Yeast extract	20
		Agar	15
3.	Potato dextrose agar (Rangaswami *et al.*, 1968)	Boiled potato - extract	250*
		Dextrose	20
		Agar	15
4.	Yeast extract agar (Rangaswami *et al.*, 1968)	Glucose	10
		Yeast extract	10
		Agar	15
5.	Nutrient agar (Rangaswami *et al.*, 1968)	Beef extract	3
		Peptone	5
		Glucose	5
		Sodium chloride	5
		Agar	15
6.	Molasses agar (Dresner, 1949)	Molasses	430*
		Sodium chloride	1.5
		Flaked Agar	20
7.	Malt extract agar (Preparation resembles yeast extract agar preparation)	Malt extract	20
		Dextrose	20
		Peptone	1
		Agar	25

* ml

Collection of fungal spores

Conidia were collected from 10-15 days old culture with the help of sterile camel hair brush or small metal spatula. The spores were stored in small tubes with screw type air tight lids and kept in an incubator at 10°C temperature and 40 per cent RH. A month old spores were used for all the experiments.

Preparation of spore suspension

The stored tubes containing spores were transferred from 10°C to 18°C and then to room temperature (25°C) to avoid the shock of sudden raise in temperature. Then spores were transferred into conical flask containing sterile distilled water along with few drops of spreader, were shaken for 10 minutes and the resulting suspension was strained through sterile double thickness cheese cloth. These processes were carried out in previously sterlized UV light chamber and care was taken to minimise the contamination.

Dilution of the spore suspension and spore count

One mg spore material obtained from culture contained about 8.0 x 10^7 spores. Thus 1.25 g of spore material obtained from culture was accurately weighed using balance 'Electron H-20' and suspended in 100 ml of water to get 10^9 spores/ml suspension. This suspension was diluted to contain 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 spores/ml by serial dilution technique. This was confirmed by using a 'Neubauer' haemocytometer (improved double ruling).

The size of the spores was measured by using a occular micrometer in a compound microscope.

Percentage of spore germination

One ml spore suspension was taken into a sterile test tube and diluted with one per cent sucrose solution to the desired concentration. A drop of this suspension was put on a cavity slide with the help of a sterile loop and covered with a round coverslip. Ringing was done with melted paraffin to avoid contamination. These slides were incubated at 30°C for 48 hr. The number of germinated spores and non-viable spores was counted and per cent germination of conidia was calculated.

Sterilization of the body surface of the insect

The eggs, larvae, pupae and moths of B. mori were surface sterilized by dipping in a sterilizing solution, either alcohol chlorine water (equal parts of 95 per cent ethyl alcohol and two per cent chlorine water) or four per cent formalin solution, for about 45 seconds. Then they were rinsed twice in distilled water for 10 seconds and allowed to dry in a previously sterilized chamber. This was done to ensure that insects are free from micro-organisms on their body surface. To separate the larvae from mulberry leaves, camel hair brush for young worms and hand with glove or chopstics for older worms were used.

Inoculation of the silkworms

After proper surface sterilization, the silkworms were inoculated with conidia of the fungus for various investigations, by any one of the following methods.

Topical application:

The external body surface of the insect was brought in contact with fungal spores by one of the following techniques.

Dipping: The surface sterilized silkworms were dipped in the spore suspension in a sterile wide beaker, for 45 seconds in case of eggs, larvae and pupae and 60 seconds in case of moths. This technique was found good as it could be done quickly as well as the conidia could be uniformly distributed all over the body surface of the insect.

Spraying: Spore suspension was sprayed on uniformly spread larvae in clean sterile tray, using a 'baby sprayer' with an automizer. Enough space was provided while spraying to prevent larvae crawling one over the other.

Pre-oral inoculation:

The mulberry leaves washed with distilled water and sterilized with 50 per cent ethyl alcohol were sprayed with the spore suspension. The leaves were allowed for water to evaporate before and after the spray. The larvae starved for 4 hr were allowed to feed on these leaves cut into small strips, taking care to see that no larvae crawled over these leaf strips to avoid cuticular contamination by the fungal spores.

The other technique adopted to administer the fungal spore suspension to anaesthesised V instar silkworm larvae through mouth was by holding them between thumb and fore finger, using a sterile blunt smooth needle in a syringe. The larval body was again sterilized with ethyl alcohol to prevent contamination of the spores in gastric fluid vomited by the larvae.

Intra-haemocoelic injection:

The V instar anaesthesised larvae were injected with spores on the right side third proleg holding between thumb and fore-finger with the help of sterile needle (26 gauge) in a syringe. About 0.3 ml of spore suspension was injected for each larvae. The prick was sealed with paraffin of low melting point to avoid bleeding.

Symptomatology of the disease

The unfertilized and dead eggs were scraped off from the egg layings on the card. The small sized, if any, were also removed to adjust the eggs in each laying to the required number. The eggs were inoculated with fungal spores by dipping. The presence or absence of the fungus on and inside the egg was confirmed by microscopic examination. The dead eggs were kept in humid chamber to observe the fungal development.

The larvae were inoculated by dipping in the spore suspension and observed for the development of external symptoms at the time of every feeding. The larvae with the black spots and red colouration among the white muscardined larvae were counted separately and expressed in per cent.

The cocoons spun by infected larvae were observed for external symptoms. They were cut open to observe the pupaeformed inside. The selected healthy cocoons were dipped in the spore suspension for two, four, six, eight and ten minutes and kept for observations.

The pupae, removed by cutting the cocoon at one end, were dipped in the spore suspension for 45 seconds and observed for external symptoms at 4 hr interval.

The moths emerged from healthy cocoons were dipped in the spore suspension for one minute with two intermittent breaks for ten seconds. The malesand females were kept in small trays separately. For observation on copulation the male and female showing sluggishness were brought together. The observations were recorded on symptoms at 2 hr interval.

Route of infection

Fifth instar silkworm larvae were inoculated with spores of B. bassiana topically (dipping), intra-haemocoelically and pre-orally. Four types of controls namely larvae untreated, dipped in sterile water, injected with sterile water into the haemocoel and fed on leaves sprayed with sterile water were kept. The larval mortality in each case was recorded.

Life cycle of the fungus

Seventy newly moulted larvae inoculated with fungal spores were kept at 80-85 per cent RH. Of which 50 were selected based on their sluggishness and fed time to time. The mortality of these larvae was recorded and cadavers were observed for sporulation of the fungus on them at 4 hr interval. The time between inoculation and complete sporulation was noted in each larvae and its average was taken as the period of life cycle (from spore to spore) of the fungus. The same procedure was followed to record the life cycle of the fungus on pupae and moths. This study was repeated in different seasons of the year and also with different races.

Effect of B. bassiana spores on different instar silkworm larvae

The different concentrations having 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 spores/ml suspension were applied separately to each instar larvae soon after their preceeding moult. The observations on daily mortality of larvae and pupae and the number of healthy cocoons formed were taken. A control with larvae treated with sterile distilled water was also kept.

Effect of *B. bassiana* spores on different silkworm races

Three concentrations having 10^3, 10^6 and 10^8 spores/ml suspension were applied separately to V instar larvae of the following silkworm races.

1. Pure Mysore (PM)
2. Hosa Mysore II (HM II)
3. C. Nichi
4. HS_6
5. NN_6D
6. NB_4D_2
7. Kalimpong A (KA)
8. J_{122}
9. PM x HS_6
10. PM x KA
11. Nandi

The total mortality of larvae and pupae and number of healthy cocoons spun by the surviving larvae were recorded.

Statistical analysis

The data on the fungal life cycle in different seasons and races and mortality of V instar larvae on different days were analysed by one way analysis and the effect of different spore concentrations on silkworm races by two way analysis of variance (Sundararaj *et al.*, 1972). The per cent larval mortality in different stages and different instars of the silkworm was analysed after arcsin transformation. The mortality in pupal stage and number of healthy cocoons formed in both cases of the effect of different spore concentrations and three spore concentrations on V instar larvae and different races of silkworm, respectively were analysed after $\sqrt{x+1}$ transformation, which was done to normalise the distribution (Snedecor and Cochran, 1967). The mortality of different larval instars of silkworm inoculated with different concentrations of B. bassiana spores was analysed by probit analysis to find out lethal concentration for 50 (LC_{50}) and 90 (LC_{90}) per cent larval mortality with five per cent fiducial limits after correcting the natural mortality by Abbot's formula (Finney, 1964). The probit analysis was also used to calculate lethal time required for 50 per cent (LT_{50}) and 40 per cent (LT_{40}) of larval mortality.

IV. EXPERIMENTAL RESULTS

The results obtained from the studies on the white muscardine disease of the mulberry silkworm, Bombyx mori L. caused by Beauveria bassiana (Balsamo) Vuillemin is presented here under different headings.

Culture of the fungus

The colony surface of all the fungal isolates in culture was creamy white in colour. The Isolates B and K showed characteristically flat, mealy, chalky and pulverent growth on all the seven media used. Where as the Isolates M and My showed tuft, cottony and elevated growth on all the media except Sabouraud's maltose agar and molasses agar in which they resembled other isolates. All the Isolates gave reddish yellow colouration to all the culture media used, but it was slight in molasses agar. This colouration was well observed by reversing the colony or by inverting the petridish with culture. The creamy white colour of the colony turned yellowish white when kept for 7-8 weeks. The culture of the Isolates B and K on Sabouraud's maltose agar with one per cent yeast kept for prolonged periods (more than 25 days) at higher (more than 80 per cent) relative humidity (RH) resulted in coremial production. This was not observed in other two isolates. Sporulation started on third day in all isolates on Sabouraud' s maltose agar with one per cent yeast. The conidia formed were chiefly globose (76.2 per cent) in shape and measured 1.5 μ to 2.9 μ (majority of them measured 2.3 μ)

in diameter. The per cent germination of spores ranged from 67 to 76 with a mean of 70.3. One mg spore material from the culture of the Isolate B contained about 10^8 spores.

Among the seven media tested for growth and sporulation of the fungus, B. bassiana, Sabouraud's maltose agar with one per cent yeast was found the best, followed by Sabouraud's dextrose agar with one per cent yeast, yeast extract agar, malt extract agar, potato dextrose agar, molasses agar and nutrient agar (Table II).

Variation in infectivity of the fungus to the silkworm

A preliminary test was conducted to study the effect of different fungal isolates on mortality of V instar silkworm larvae. The spore suspension of the Isolates B, K, M and My was sprayed separately to four lots of silkworm larvae, resulted in 100.00, 96.33, 81.12 and 80.33 per cent larval mortality, respectively.

Symptomatology of the white muscardine disease

Egg:

The colour of the infected eggs turned somewhat pale or pink after 4-5 days of inoculation, looked partially filled and the chorion shrunk as compared to healthy eggs. The presence of the fungal mycelium on the surface and inside the dead eggs was detected with the help of a microscope as it could not be markedly seen by the naked eye. The fungus

Table II. Effect of different media on the growth and sporulation of different isolates of Beauveria bassiana*

Medium	Isolate B		Isolate K		Isolate M		Isolate My	
	1	2	1	2	1	2	1	2
Sabouraud's maltose agar with one per cent yeast	++++	++++	++++	+++	++++	++	++++	+
Sabouraud's dextrose agar with one per cent yeast	+++	+++	+++	++	++++	++	+++	+
Potato dextrose agar	++	++	++	+	++	+	++	-
Yeast extract agar	+++	+++	+++	++	+++	+	+++	+
Nutrient agar	+	+	+	+	+	-	+	-
Molasses agar	++	+	+	+	+	-	++	-
Malt extract agar	+++	+++	+++	++	+++	+	+++	+

* Observed on 13th day of inoculation of the culture medium kept at 90 per cent relative humidity and 25-27°C temperature.

1 Mycelial growth

2 Sporulation

++++	Luxurient
+++	Better
++	Little
+	Negligible
-	None

did not extend outside the chorion of the egg to produce conidia at room temperature and RH, even a week after the death of the egg.

Larva:

The larvae became somewhat sluggish from 2.0 to 2.5 days of inoculation with fungal spores. Soon after they lost the usual shining nature of the skin and showed less appetite. As the disease advanced, the larvae failed to respond readily to external stimuli like light, heat or irritability to touch or pricks of the needle. Later the larvae turned slightly pale in colour and became almost inactive due to paralysis. By this time the larvae fed very little and remained underneath the fresh leaves provided.

The larvae lost their elasticity and became limp, exhibited no spontaneous movements and lost their irritability completely. On careful observation the larvae showed areas around the spiracles slightly brown in colour. Later they developed into black or brown patches, first ventrally, laterally and then dorsally, especially on the intersegmental regions. They were transformed into oily dark brown patches (Fig. 1) in 4.0 to 5.5 days after inoculation in the V instar and 2.5 to 4 days in other instars. Some muscardined larvae exhibited no spots, but majority of them showed 5 to 22 black spots. The larvae showing black spots were 10.75, 23.25, 38.50, 63.25 and 76.50 per cent in I, II, III, IV and V instars, respectively. The microscopic examination revealed

Fig. 1. Muscardined larvae showing oily black or brown patches.

Fig. 1

the presence of branched and free mycelia in the haemolymph and branched mycelia in the integument with black spot in addition to the abundant spores of the fungus.

The infected larvae stopped feeding from 4-6 days after inoculation and started vomiting gastric juice. The gut content became more watery and the larvae were soft and flobby to touch followed by frequent diarrhoea with agony in the beginning which became severe just before death. The larval excreta was in the liquid form, if hard, the excretory pellets were found connected to each other with excretory fluid giving the appearance of a beaded string. No external deformations were observed. The death occurred any time between 4 and 11 days after inoculation in case of V instar and 3 and 6 days in other instars.

The cadaver was soft and pliable, shrunk laterally or dorsally and the hardening started generally from posterior end. Majority of the dead larvae (77.05 per cent) showed red or pink colouration (Fig. 2a) in 6-8 hr of death and a few others did not show any red colouration. The red colouration was 63.00, 60.00, 77.50, 89.00 and 95.75 per cent in I, II, III, IV and V instars, respectively (Table III).

The corpses became rigid, hard and the white efforescence started first on black spots, around spiracles and intersegmental regions towards the posterior end (Fig. 2b) and subsequently spread all over the corpses (Fig. 2c).

Table III. Mean per cent larvae* of Bombyx mori L. showing black spots and red colouration.

Sl. No.	Instar	Black spots	Red colouration
1.	I	10.75	63.00
2.	II	23.25	60.00
3.	III	38.50	77.50
4.	IV	63.25	89.00
5.	V	76.50	95.75

* Based on four replications each with 100 muscardined larvae.

It was completely covered with conidial felt in 1.5 to 2.5 days at 80-85 per cent RH or 15-20 hr at 95-100 per cent RH, when the temperature remained between 25 and 27°C. Ultimately it was transformed into mummies which gave the appearance of a 'lime covered stick' or 'birds excreta' with loosely attached fine white powder (Fig. 2d, 3), which contained mainly conidia of the fungus and negligible amounts of its mycelia. No coremia, perithecia and white crystals on the surface and sclerotia inside the corpses were observed. The mummified cadavers became brittle and could be crumbled into pieces or fine white powder like 'lime'. No decomposition of the corpses was observed during the course of the study.

All the symptoms mentioned above were observed in all the instars (Fig. 3). Generally, infected larvae failed to spin the cocoon completely. They stopped spinning and died thus resulting in one fourth and half spun cocoons (Fig. 4).

Cocoon:

The cocoons spun by the infected worms did not exhibit any external symptoms. The muscardined cocoons kept for 4-5 days after death sounded like oven dried ones, when shaken. When these were cut open, the pre-pupae or pupae were found dead and completely covered with white efforescence (Fig. 5). These infected cocoons were lighter and no moths emerged from them. Healthy cocoons dipped in the spore suspension for 2 t 10 minutes did not produce any symptoms of the disease.

Fig. 2. The symptoms of the white muscardine disease after the death of the larvae.

a) Pink colouration of larval cadaver.
b) White efforescence on intersegmental regions.
c) White efforescence all over the cadaver.
d) Mummified 'lime covered stick' like cadaver.

Fig. 3. 'Lime covered stick'like appearance of I, II, III, IV and V instar larvae, respectively.

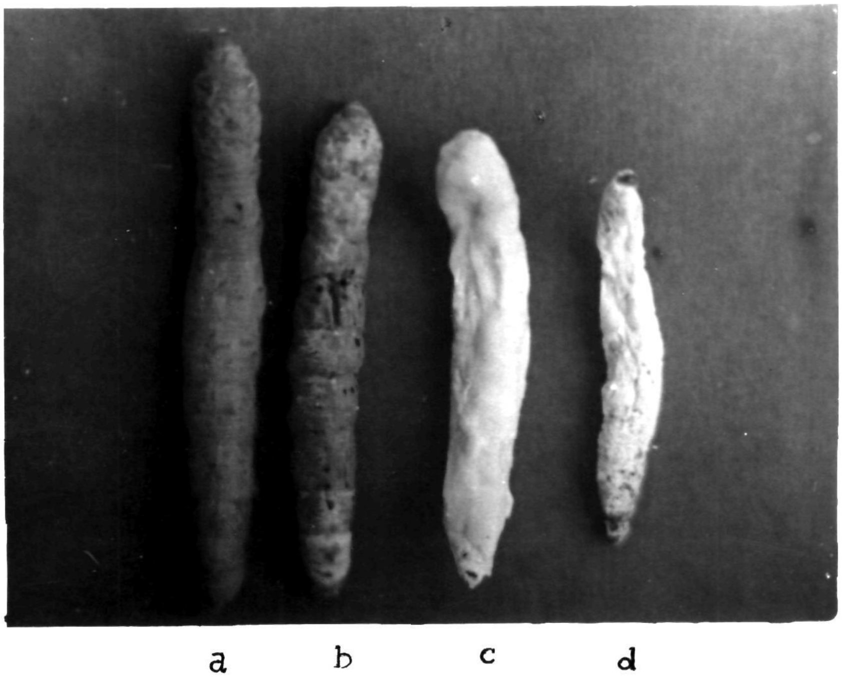

Fig. 2

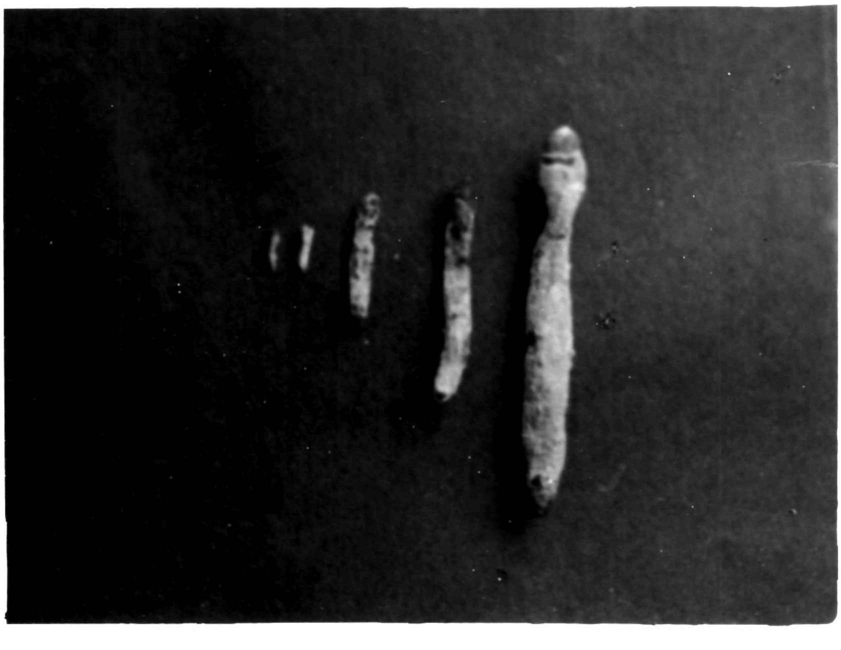

Fig. 3

Fig. 4. One-fourth and half spun cocoons by the infected larvae.

Fig. 5. The dead pre-pupae showing the white efflorescence inside the cocoons.

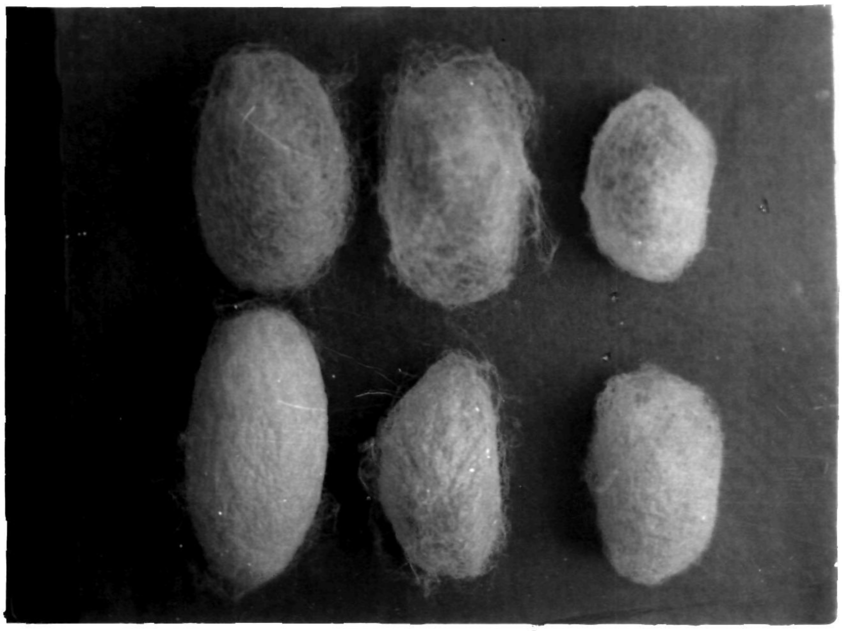

Fig. 4

Fig. 5

The naked pupae, taken out from healthy cocoons and dipped in the spore suspension, showed symptoms of the disease in a week. They became soft and dark especially on the intersegmental regions and showed signs of uneasiness in 2-3 hr just before death which occurred in between 9 and 11 days of inoculation.

Later, in 4 hr, their bodies were hardened and the white efforescence started on the intersegmental regions, first on the ventral side (Fig. 6b) and later on the dorsal surface (Fig. 6a), which was confined to intersegmental regions only under room conditions. It covered the whole cadaver in about 36 hr (Fig. 6c) when kept at 90-95 per cent RH.

Moth:

Infected moths became sluggish with slightly crinkled wings (Fig. 7) and evinced less interest in copulation. Females compared to males showed least interest in copulation. The sluggish males did not reject females, when provided, for copulation. The fungus was found concentrate more on the reproductive organs like ovary in females and testis in males although it was found in all over the body cavity. The death occurred in both sexes in 48-65 hr after inoculation. Similar to the symptoms on the larval cadaver, the cadavers of the moths hardened and showed red colouration, which was very clear all over the body of the moth when the scales were removed. Within 4-8 hr after death of the moth the white

Fig.6. White efforescence on the pupal cadavers.

Fig.7. The cadavers of females and male moths.

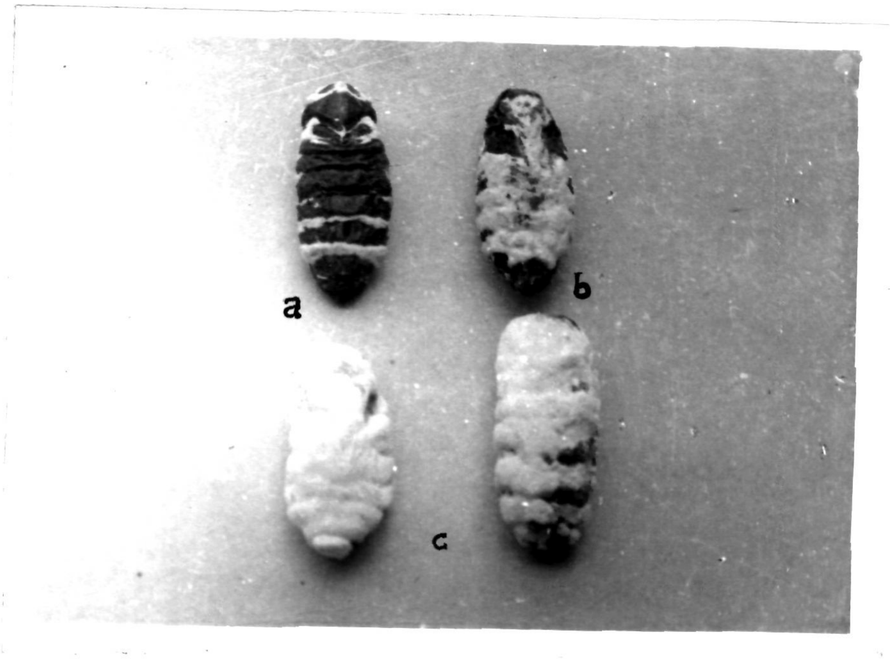

Fig. 6

Fig. 7

efforescence started on intersegmental regions of the abdomen (Fig. 7). It did not spread to segmental regions except the lateral regions under room conditions. When these muscardined moths were kept at 85-90 per cent RH, the entire body of the moth including first half of the wings became covered with white efforescence (Fig. 7).

Route of infection

The results of the experiment conducted to see the mortality of larvae when the pathogen was introduced into the body of the V instar larvae through different routes is presented in Table IV. The highest mean larval mortality was obtained in larvae injected with spore suspension into the haemocoel (47.75), followed by topical application (43.75) and oral inoculation (27.50).

Life cycle of the fungus, *B. bassiana*

The life cycle of the fungus in larva (V instar), pupa and moth on an average took 6.10, 10.5 and 2.65 days with a range of 4.5 to 10.5, 9.0 to 12.5 and 2.0 to 3.5 days, respectively. The period of life cycle of the fungus in different larval instars, V instar larvae during different rearing seasons of the year 1976-77 and V instar larvae of different silkworm races is presented in Tables V, VI and VII, respectively.

Table IV. Mean mortality of Bombyx mori L. larvae inoculated with Beauveria bassiana spores by different methods.

Sl. No.	Method of application	Mean mortality*	Transformed ($\sqrt{x+1}$) mean
1.	Larvae dipped in the spore suspension.	43.25	6.65
2.	Larvae injected with spores suspension into its haemocoel.	47.75	6.98
3.	Larvae fed with leaves sprayed spore suspension.	27.50	5.33
4.	Control:		
	a) Larvae dipped in sterile water	0.25	1.10
	b) Larvae injected with sterile water	0.50	1.18
	c) Larvae fed with leaves sprayed with sterile water	0.00	1.00
	d) Larvae left untreated	0.00	1.00

* Based on four replications each with 50 larvae.

CD (P.=0.05) = 3.18

(P.=0.01) = 4.33

Table V. Life cycle of Beauveria bassiana (Bals.) Vuill. in different instar silkworm larvae (in days).

Sl. No.	Larval instar	Range	Mean
1.	I	3.0-4.0	3.69
2.	II	2.5-3.5	3.00
3.	III	4.0-5.0	4.50
4.	IV	5.5-6.5	6.00
5.	V	5.5-6.5	6.13

Table VI. Life cycle of <u>Beauveria bassiana</u> (Bals.) Vuill. in V instar silkworm larvae, during different rearing seasons of the year 1976-77 (in days).

Sl. No.	Rearing season	Mean*
1.	July-August 1976	6.25
2.	August-September 1976	5.81
3.	September-October 1976	7.19
4.	October-November 1976	10.88
5.	November-December 1976	11.56
6.	January-February 1977	8.94
7.	March-April 1977	6.13
8.	May-June 1977	3.88
9.	June-July 1977	4.56

* Based on four replications each with 25 larvae.

Table VII. Life cycle of the fungus in V instar larvae of different races of the silkworm (in days).

Sl. No.	Race	Mean*
1.	Pure Mysore	7.125
2.	Hosa Mysore II	6.188
3.	C. Nichi	5.938
4.	HS_6	5.250
5.	NN_6D	6.250
6.	NB_4D_2	6.000
7.	Kalimpong A	6.250
8.	J_{122}	5.938
9.	Pure Mysore x HS_6	6.125
10.	Pure Mysore x Kalimpong A	6.250
11.	Nandi	5.750

* Based on 4 replications, each with 50 infected larvae.

$F = 11.856$

$CD_{(P.= 0.05)} = 0.37$

$S_{Em} = 0.135$

Mortality of the silkworm

The mortality of the silkworm inoculated with the fungal spores in different stages is presented in Table VIII.

The freshly laid eggs were found to be more susceptible (8.13 per cent mortality) than 5-6 days old (6.6 per cent mortality) and 6-7 days old (5.7 per cent mortality) eggs. The preliminary studies on the pupae inoculated with spores showed that the younger (3-5 days old) pupae were more susceptible than older (6 days old) ones as the latter gave normal moth emergence.

The virgin moths were less susceptible than the mated ones. The mated females were comparatively less susceptible than females which already laid their eggs. The former ones laid more eggs (on an average 256.3 eggs) as compared to the females mated after inoculation (on an average 101.5 eggs) and healthy ones (on an average 513.7 eggs).

The per cent mortality of silkworm in different larval instars and V instar larvae inoculated with B. bassiana spores on different days is given in Table IX and XI, respectively. It was 69.25 and 70.25 in larvae inoculated with spores a day before and during the IV moult. The results of experiments conducted to see the effect of B. bassiana spores on different instar larvae, including the effect in the succeeding instars and the total number of healthy cocoons spun is

Table VIII. Per cent mortality of different stages of silkworm Bombyx mori L. after inoculation with Beauveria bassiana spores.

Stage	Per cent mortality*	Arcsin mean
Egg	5.7	13.67
Larva	99.9	88.20
Pupa	40.9	39.74
Moth	78.1	62.10

* Based on five replications each with 400 individuals.

Arcsin $CD_{(P.=0.05)}$ = 14.768

$(P.=0.01)$ = 20.350

Table IX. Per cent mortality of different instar *Bombyx mori* larvae inoculated with *Beauveria bassiana* spores.

Instar	Per cent mortality*	Arcsin mean
I	45.92	42.66
II	36.25	27.02
III	60.20	50.91
IV	88.67	70.39
V	99.55	85.96

* Based on four replications each with 300 larvae.

Arcsin CD$_{(P.= 0.05)}$: 2.131
$(P.= 0.01)$: 3.313

able X. Effect of <u>Beauveria</u> <u>bassiana</u> spores on the silkworm, <u>Bombyx</u> <u>mori</u> larvae inoculated in different instars*.

nstar nocu-ated	No. of larvae dead						Pupal mort-ality	No. of healthy cocoons spun
	I instar	II instar	III instar	IV instar	V instar	Ripe		
I	310 (38.75)	45 (5.63)	82 (10.25)	118 (14.75)	132 (16.50)	0 (0.00)	10 (1.25)	103 (12.88
II	-	131 (16.38)	192 (24.00)	121 (15.13)	258 (32.25)	8 (1.00)	21 (2.63)	69 (8.63
III	-	-	304 (38.00)	184 (23.00)	267 (33.38)	23 (2.88)	4 (0.50)	18 (2.25
IV	-	-	-	669 (83.63)	99 (12.38)	14 (1.75)	12 (1.50)	6 (0.75
V	-	-	-	-	714 (89.25)	69 (8.63)	17 (2.13)	0 (0.00

* Based on four replications each with 200 larvae and figures in parantheses shows the percentage.

Table XI. Mortality of silkworm larvae treated with spores on different days soon after the IV moult.

Sl. No.	Day after IV moult	Per cent mortality*
1.	I	71.50
2.	II	68.00
3.	III	66.75
4.	IV	65.25
5.	V	64.25
6.	VI	60.00
7.	VII	55.50

* Based on four replications each with 100 larvae.

F = 5.46

$CD_{(P.=0.05)}$ = 87.95

presented in Table X. The I, II, III, IV and V instar larvae treated with spores showed the per cent mortality of 38.75, 16.38, 38.00, 83.63 and 89.25, respectively and the per cent of healthy cocoons spun by the survived larvae was 12.88, 8.63, 2.25, 0.75 and 0.00, respectively.

Effect of different concentrations of B. bassiana spores on the silkworm, B. mori

The data on the effect of different concentrations of B. bassiana spores on different larval instars, pupae and cocoons spun are presented in Appendix I. The larval mortality of the larvae treated with 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 spores/ml suspension along with provisional probits (from Fig. 8) to calculate the lethal concentration of spores for 50 per cent larval mortality (LC_{50}) is given in Appendix II. The analysis of the data obtained in the experiment is presented in Table XII.

To obtain the larval mortality in I, II, III, IV and V instars of 50 per cent, the concentration of spores required was 1598, 1410, 1171, 602 and 435, respectively. This (LC_{50}) and lethal concentration of spores for 90 per cent mortality (LC_{90}) along with their fiducial limits at 5 per cent level and other particulars are given in Table XII.

The lethal time required for the per cent mortality of the larvae, of 50 (LT_{50}) and 40 (LT_{40}), treated with 10^3 spores of B. bassiana per ml suspension (from Fig. 9) is

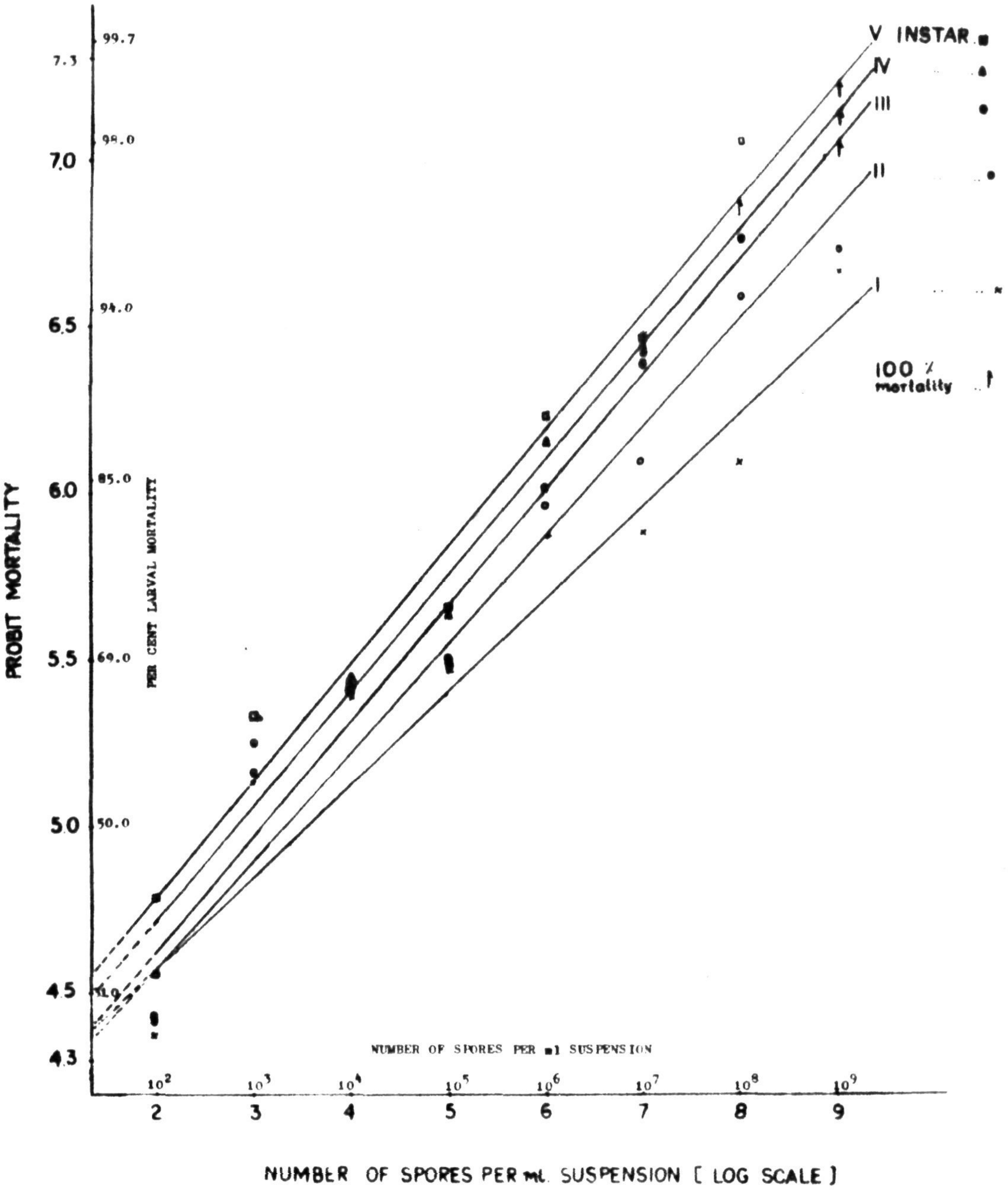

FIG 8. CONCENTRATION-MORTALITY RESPONSE OF DIFFERENT INSTAR SILKWORM, *B MORI* LARVAE TO VARIOUS CONCENTRATIONS OF *BEAUVERIA BASSIANA* SPORES.

Table XII. Probit analysis of the Concentration-Mortality response of different larval instars of the silkworm, *Bombyx mori* L. inoculated with different concentrations of *Beauveria bassiana* spores.

Particulars		Larval instars				
		I	II	III	IV	V
'a' value		4.41055	4.02651	3.91778	3.91778	4.02020
'b' value (slope)		0.26829	0.30913	0.35265	0.36516	0.37136
Standard error of 'b'		0.00852	0.00934	0.01002	0.01029	0.01061
'g' value		0.00390	0.00350	0.00310	0.00305	0.00314
LC_{50}		3.20351 (1598.0)	3.14916 (1410.0)	3.06885 (1171.0)	2.77930 (601.6)	2.63845 (435.0)
Fiducial limit for LC_{50} at P. = 0.05	Upper:	3.37564 (2374.0)	3.30186 (2004.0)	3.20582 (1606.0)	2.92010 (832.0)	2.78061 (603.4)
	Lower:	3.03140 (1174.0)	2.99650 (991.9)	2.93187 (854.9)	2.63850 (435.0)	2.49630 (313.5)
LC_{90}		7.98052 (95610000)	6.76000 (5754000)	6.70310 (5048000)	6.28901 (1945000)	6.08960 (1229000)
Fiducial limit for LC_{90} at P. = 0.05	Upper:	8.09885 (152600000)	6.90235 (7987000)	6.86420 (7314000)	6.43588 (2729000)	6.23176 (1703000)
	Lower:	7.86220 (72810000)	6.61766 (4147000)	6.54203 (3483000)	6.14215 (1389000)	5.94744 (348300)

Figures in parantheses show the actual number of spores.

Fiducial limits were calculated by using normal equivalent deviate (P. = 0.05), 1.96.

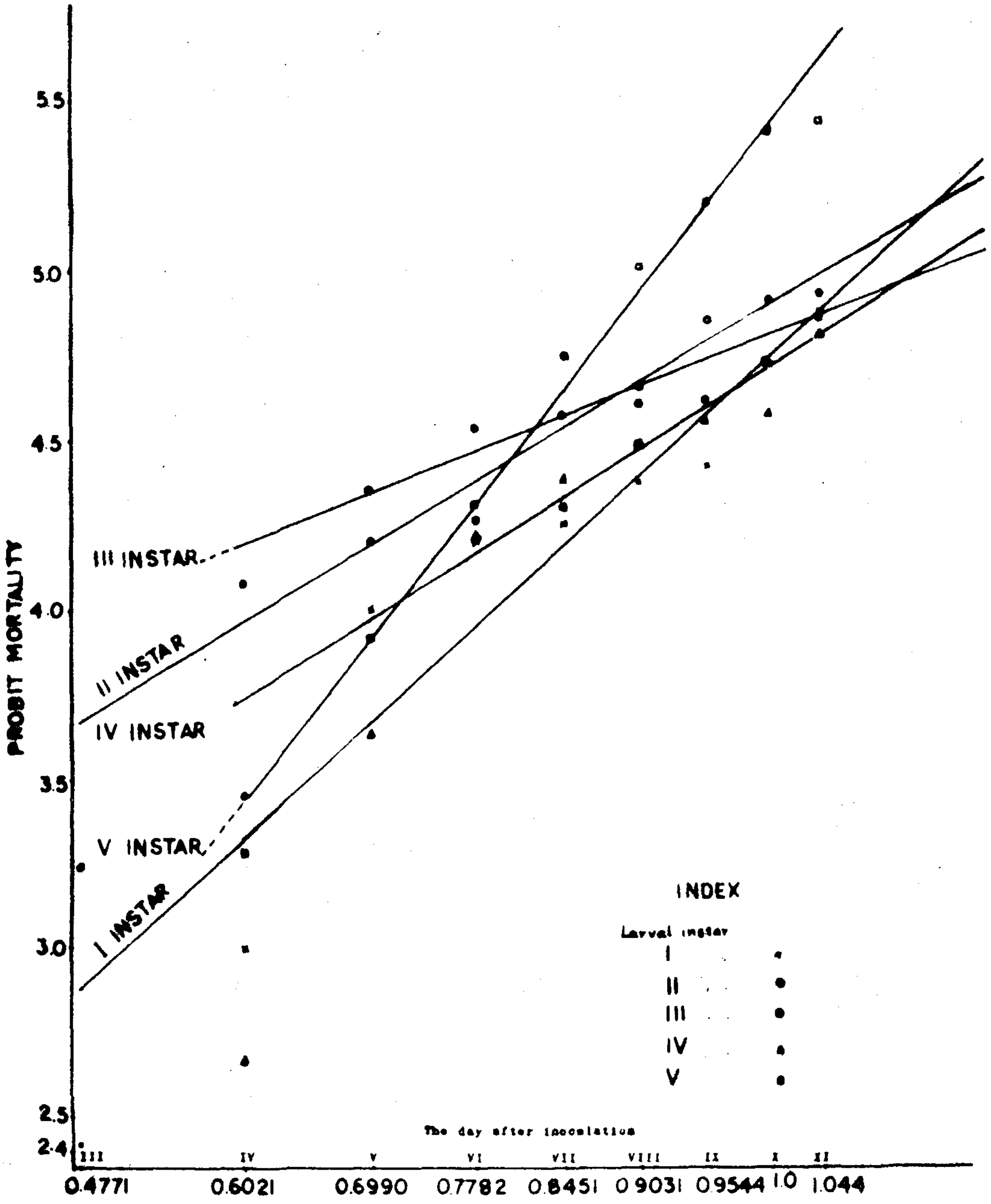

FIG. 9. TIME-MORTALITY RESPONSE OF DIFFERENT INSTAR SILKWORM B. MORI LARVAE TO 10^3 SPORES OF BEAUVERIA BASSIANA PER ML SUSPENSION.

presented in Table XIII. The median lethal time (LT_{50}) of IV (from Fig. 10) and V (from Fig. 11) instar larvae at different concentrations of B. bassiana spores is given in Table XIV.

The data on pupal mortality due to the inoculation of B. bassiana spores to larvae is presented in Table XV. The number of healthy cocoons formed out of inoculated larvae with different spore concentrations is given in Table XVI.

Effect of B. bassiana on different silkworm races

The data on the effect of B. bassiana on larva and pupa of different races of silkworm when the larvae inoculated with 10^8, 10^6 and 10^3 spores/ml suspension is presented in Table XVII.

The Pure Mysore race showed least larval mortality and J_{122} race showed the highest. The per cent of healthy cocoons spun by the survived larvae was highest in Pure Mysore at 10^3 and 10^6 spore concentration and C. Nichi at 10^8 spore concentration and least in J_{122} race at all the spore concentrations used. The minimum pupal mortality per cent of 0.5 was obtained in races HM II, NB_4D_2 and Pure Mysore x Kalimpong A and maximum of 3.25 in C. Nichi.

The per cent of larval mortality and cocoons spun by the survived larvae in different races of silkworm inoculated with B. bassiana spores is shown in Fig. 12.

Table XIII. Lethal time required for different larval *Bombyx mori* instars at 10^3 spores of *Beauveria bassiana* per ml suspension (in days).

Instar	LT_{50}*	LT_{40}*
I	11.890	10.060
II	11.150	8.660
III	13.570	9.173
IV	13.180	11.610
V	8.269	7.286

* From Fig. 9

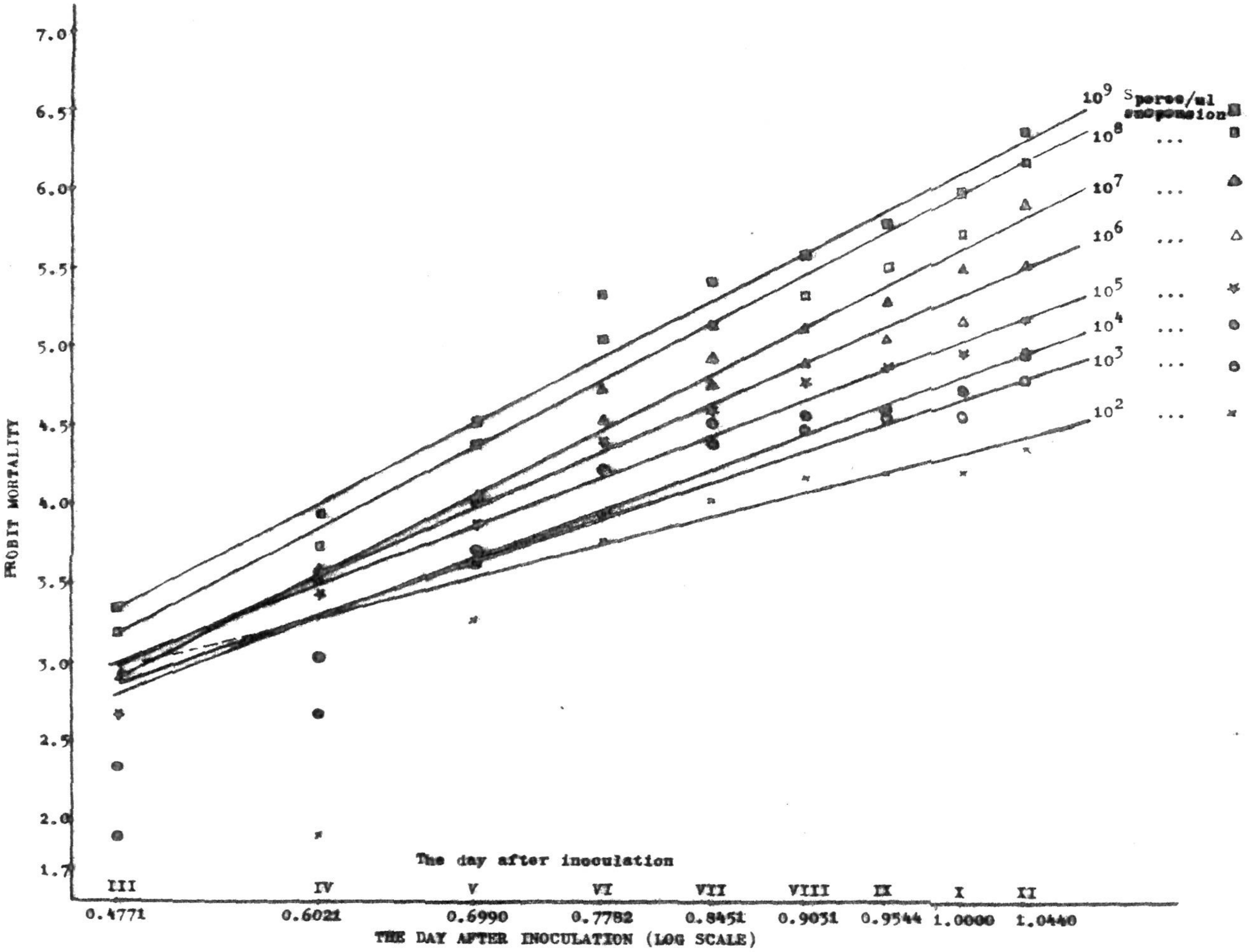

FIG.10. TIME-MORTALITY RESPONSE OF IV INSTAR SILKWORM, *BOMBYX MORI* LARVAE TO DIFFERENT CONCENTRATIONS OF *BEAUVERIA BASSIANA* SPORES.

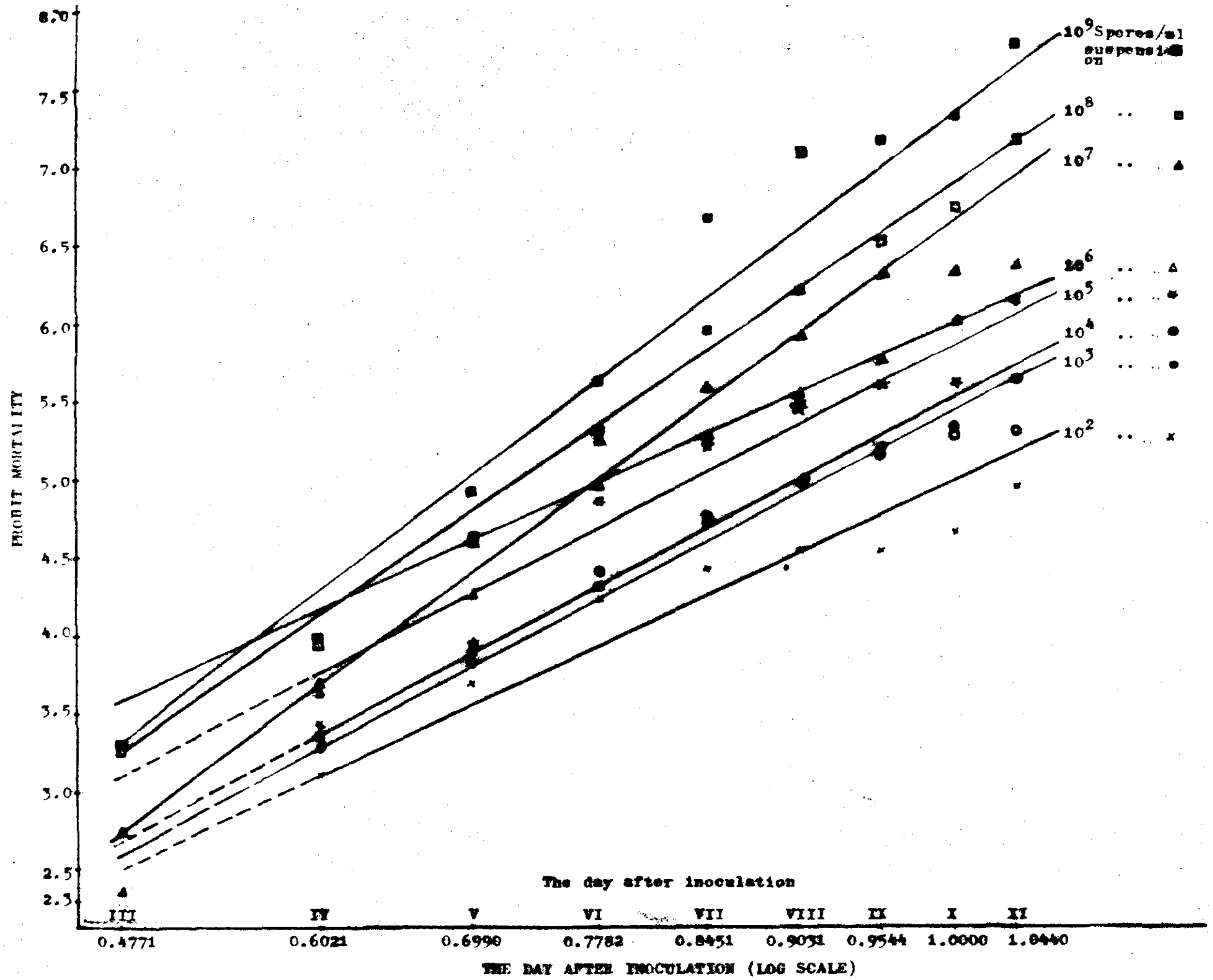

FIG.11. TIME-MORTALITY RESPONSE OF V INSTAR SILKWORM <u>BOMBYX MORI</u> LARVAE TO DIFFERENT CONCENTRATIONS OF <u>BEAUVERIA BASSIANA</u> SPORES.

Table XIV. Median lethal time (LT_{50}) for IV and V instar silkworm larvae at different concentrations of Beauveria bassiana spores (in days).

No. of spores/ml suspension	IV instar*	V instar**
10^9	6.131	4.955
10^8	6.569	5.309
10^7	7.586	6.001
10^6	8.463	6.060
10^5	9.716	6.839
10^4	11.150	8.000
10^3	13.180	8.269
10^2	-	9.942

* From Fig. 10

** From Fig. 11

Table XV. Per cent mortality of pupae formed out of 800 larvae of different instars treated with different concentrations of <u>Beauveria bassiana</u> spores.

Sl. No.	No. of spores/ml suspension	I instar	II instar	III instar	IV instar	V instar
1.	10^9	0.75	1.38	0.00	0.00	0.00
2.	10^8	0.50	0.63	1.50	0.13	0.00
3.	10^7	2.13	0.38	1.13	0.50	0.25
4.	10^6	1.25	0.50	1.13	0.50	0.38
5.	10^5	0.38	0.75	0.63	0.25	0.00
6.	10^4	0.38	0.63	0.63	0.13	1.00
7.	10^3	1.25	0.75	0.63	0.13	0.50
8.	10^2	0.00	0.50	0.50	1.13	0.13
9.	Control	0.00	0.00	0.00	0.00	0.00
*CD (P.=0.05)		0.59	0.69	0.45	0.50	0.57
(P.=0.01)		0.79	0.94	0.87	0.68	0.77

* in $\sqrt{x+1}$ transformation

Table XVI. Per cent of healthy cocoons formed out of 800 larvae of different instars treated with different concentrations of *Beauveria bassiana* spores.

Sl. No.	No. of spores/ml suspension	I instar	II instar	III instar	IV instar	V instar
1.	10^9	3.75	2.50	0.00	0.00	0.00
2.	10^8	12.63	5.00	3.13	1.13	0.00
3.	10^7	15.88	12.88	8.50	7.00	6.63
4.	10^6	17.00	15.88	13.75	11.63	10.38
5.	10^5	30.50	30.00	29.38	25.50	25.25
6.	10^4	33.50	33.00	32.75	32.50	32.25
7.	10^3	41.75	41.63	38.38	36.50	36.25
8.	10^2	71.38	69.50	69.25	65.00	59.88
9.	Control	96.88	97.13	97.38	98.50	98.38
*CD (P.=0.05)		0.51	0.49	0.41	0.82	0.33
(P.=0.01)		0.69	0.66	0.56	1.10	0.44

* in $\sqrt{x+1}$ transformation

Table XVII. Effect of three concentrations of *Beauveria bassiana* (Bals.) Vuill. spores on the larvae and pupae of different races of silkworm, *Bombyx mori* L. (out of 400 larvae in each concentration).

Sl. No.	Race	No. of larvae died			No. of pupae died			No. of healthy cocoons formed			No. of individuals died of other causes		
		10^8	10^6	10^3	10^8	10^6	10^3	10^8	10^6	10^3	10^8	10^6	10^3
1.	Pure Mysore	344	201	62	9	22	6	37	153	330	8	24	4
2.	Hosa Mysore II	391	374	187	2	6	8	2	17	192	5	3	13
3.	C. Nichi	343	246	87	13	8	2	38	142	308	6	2	3
4.	HS_6	375	338	172	5	8	4	11	49	220	9	4	4
5.	NN_6D	373	360	195	8	5	8	7	31	193	12	4	4
6.	NB_4D_2	382	374	219	2	0	6	5	21	171	11	4	4
7.	Kalimpong A	385	364	192	4	8	6	7	26	185	4	2	7
8.	J_{122}	392	392	260	1	3	5	0	3	132	7	2	3
9.	Pure Mysore x HS_6	369	306	170	7	3	1	19	84	222	5	7	7
10.	Pure Mysore x Kalimpong A	386	344	229	2	8	2	7	38	169	3	10	0
11.	Nandi	379	338	193	5	3	5	13	48	201	3	11	2

'F' values:	No. of larvae died	No. of pupae died	No. of healthy cocoons formed
Races:	25.83	68.56*	1.88*
Concentrations:	524.32	1629.46*	9.87*
Interaction:	3.09	4.33*	1.40*

(* in $\sqrt{x+1}$ transformation)

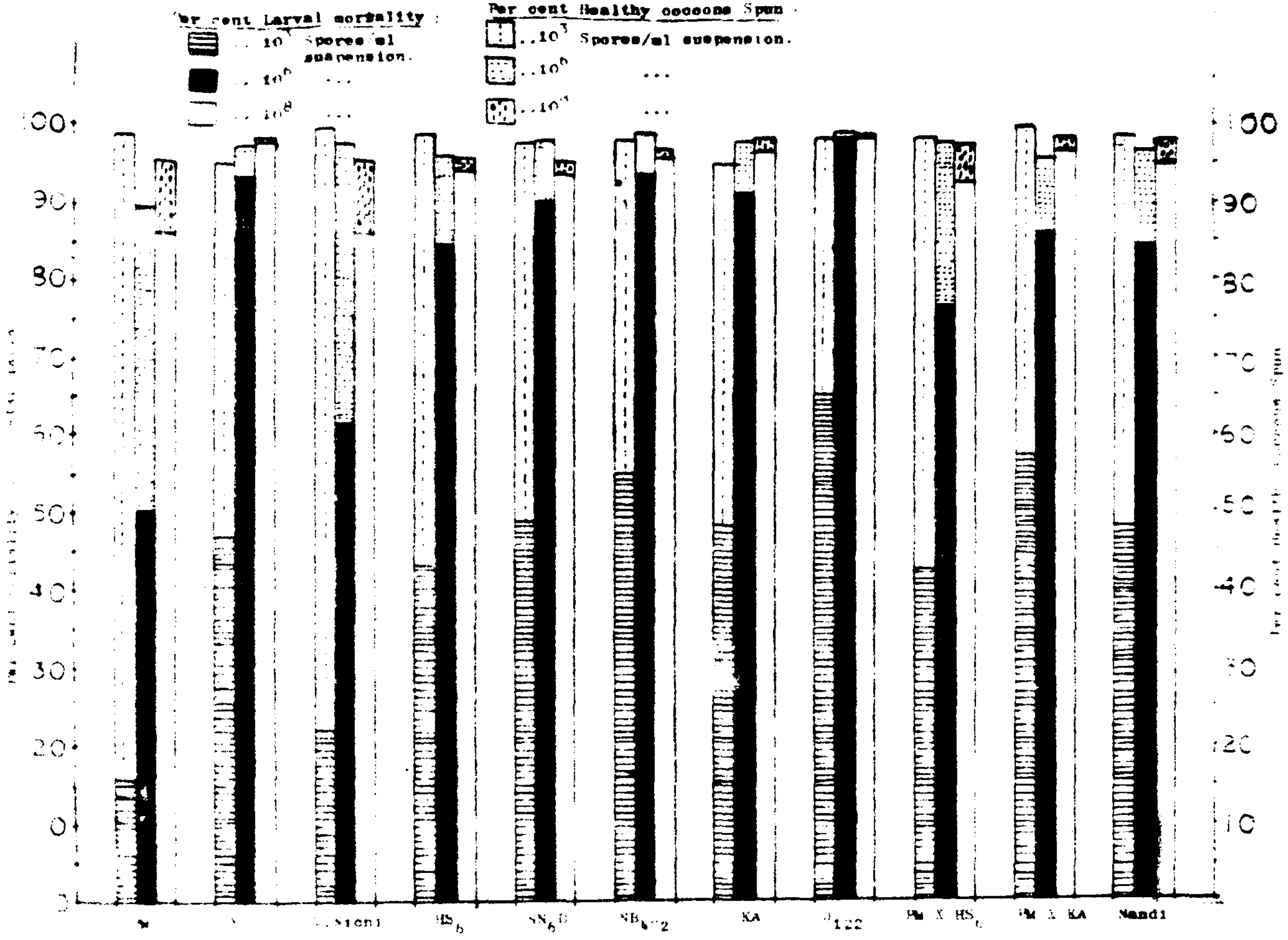

FIG 12 PERCENT OF LARVAL MORTALITY AND COCOONS SPUN BY DIFFERENT SILKWORM RACES INOCULATED WITH THREE CONCENTRATIONS OF *BEAUVERIA BASSIANA* SPORES.

DISCUSSION

V. DISCUSSION

The available literature on white muscardine disease of the mulberry silkworm, Bombyx mori L. caused by Beauveria bassiana (Balsamo) Vuillemin is although exhaustive still many basic aspects of it are not clearly understood. Hence, the present preliminary studies were undertaken, with the hope that it might help in the better management of the disease. The results of the studies are discussed hereunder in the light of the work on insect pathology, especially on mycoses, carried out by several workers all over the world.

Culture of the fungus

The colony of the fungus was creamy white with characteristic flatty, mealy, chalky and pulverent growth in case of the Isolates B and K and tuft, cottony and elevated growth in case of the Isolates M and My. The colony characters of the Isolates B and K resembled the colony characters B. bassiana and that of Isolates M and My resembled somewhat B. globulifera (Lefebvre, 1931).. The latter was considered as a strain of B. bassiana (MacLeod, 1954a). Thus Isolates M and My could be mild B. bassiana globulifera. The cultural variation in different strains of B. bassiana similar to the present observations was reported by MacLeod (1954b). MacLeod (1954a, b), Urs et al. (1965) and Thontadarya (1966) have reported similar characters of B. bassiana colonies as found for the Isolates B and K of B. bassiana in the present studies.

All the isolates gave reddish-yellow colouration to all the seven media used for its culture as reported by Siemaszko (1937), Lefebvre (1931) and MacLeod (1954a). The colouration of the culture media could be said to be due to the fungus itself as it was seen in pure culture also. Siemaszko (1937) has stated that the colour was due to the association of bacterium, Serratia marcescens Bizio, but MacLeod (1954a) disputed this, after thorough investigations on various aspects of the fungus, B. bassiana.

The creamy white colour of the colony surface changed to yellowish-white after 7-8 weeks of storage. Thontadarya (1966) has reported a similar change in 10-12 weeks of storage. All the Isolates found to sporulate on third day(68 hr) after inoculation on Sabouraud's maltose agar with one per cent yeast. Sporulation within 2-4 days is also reported by Lefebvre (1934), Urs et al. (1965), Thontadarya (1966), Samsinakova and Misikova (1973) and Rao (1975).

The diameter of the conidia ranged from 1.5 to 2.9 µ. Various conidial diameters, have been reported by several workers, as 2.0 to 2.5 (Delocroix, 1893), 1.5 to 3.0 (Ferraris, 1910; Dieuziede, 1925), 2.1 to 2.5 (Lefebvre, 1931), 1.5 to 2.0 (Petch, 1931), 1.9 to 3.3 (Siemaszko, 1937), 1.0 to 4.0 (MacLeod, 1954a) and 1.5 to 2.25 (Urs et al., 1965) microns. The present findings on the spore diameter agree with most of the workers. All these workers have reported the shape of the spores as globose except Lefebvre (1931) and Petch (1931) who found in addition the oval shaped spores, but the globose spores were in larger numbers. The present investigations with respect to shape of the spores agree with that of Lefebvre (1931) and Petch (1931).

One mg spore material contained about 10^8 spores. This is close to the findings of Muller-Kogler (1967) that 85×10^6 spores/mg in case of B. tenella and Thontadarya (1966) who reported 2.05×10^8 and 2.10×10^8 spores/mg for strain 3108 and 4007 of B. bassiana, respectively. The germination of spores on an average was 70.3 per cent. As high as 98 per cent spore germination has been reported by many workers. The low percentage of germination obtained in the present study may be due to environmental factors (Walstad et al., 1970). The coremial production observed in Sabouraud's maltose agar culture kept for prolonged periods was also reported previously (Dieuziede, 1925; MacLeod, 1954a; Thontadarya, 1966).

Sabouraud's maltose agar with one per cent yeast was found to be the best of all seven media used for culture of this fungus (Table II). This finding confirms the observations made by MacLeod (1954a) and Samsinakova (1966). Thomas (1974) also recommended this media for culturing the fastidiously growing entomopathogenic fungi. The other media did not yield larger quantities of spores of B. bassiana.

Variation in infectivity of the fungus to the silkworm

The four Isolates of the fungus, *B. bassiana* inoculated to the *B. mori* larvae gave different mortality per cents. Among the Isolates, Isolate B produced higher larval mortality (100 per cent) than other isolates. This indicates the existence of strains which differ in their virulence. Variation in virulence among different biotypes of the fungus towards a specific host insect was previously reported by Fargues (1972) and Thontadarya (1966) who found the difference between two strains of *B. bassiana* in the infectivity to the rice weevil, *Sitophilus oryzae* (L.) and cowpea weevil, *Callosobruchus maculatus* (Fabricius).

Symptomatology of the white muscardine disease

The interaction of a host, pathogen and environment results in the disease development being manifested by a number of symptoms or signs which are characteristic of a particular disease. These symptoms may be classified into external, behavioural, physiological and anatomical (Madelin, 1963). In the present studies on external and behavioural symptoms were studied to form keys for easy identification of the disease at different stages of the silkworm.

Egg:

The infected eggs could be easily recognized as they were pale or pink, partially filled and the chorion shrunk.

This seems to be the first report of changed colour of infected silkworm eggs. Medelin (1963) has also reported the distinct symptoms of fungal diseases of insect eggs, in general. But Steinhaus (1965) has reported that the pathologies of infected eggs is almost non-existent. The eggs of the beetle, *Melolontha melolontha* L. infected with *B. tenella* turned pink or red (Hurpin and Vago, 1958) and *Metarrhizium anisopliae* turned brown in colour with dried up appearance (Blunk, 1939). This colouration is definitely due to the fungus itself as its secretions contain red or yellow colour pigments (El Basyouni *et al*., 1968). Madelin (1963) has also stated that the change in colour of the eggs infected with entomopathogenic fungi was not rare. The infected eggs of silkworm exhibited emptyness and partially collapsed chorion as in case of *Galleria mellonella* L. eggs infected with *Aspergillus flavus* (Behnke and Yendol, 1969).

In the present study mycelium was noticed on the surface and inside the infected eggs. Similar observations were made by Behnke and Yendol (1969) who have further reported that the mycelium could penetrate the chorion, but with little external growth of the fungus, *A. flavus* on greater wax moth egg.

Larva:

Sluggishness, loss of irritability and elasticity, appearance of black patches on the body before death and red or pink colouration of the body, white efforescence over the body after death of the larvae are some of the symptoms of the muscardine(Fig. 1, 2, 3). This was clearly observed in all the instars especially V instar larvae. The faded colour of the infected larvae observed has been previously reported (Mukerji, 1912; Tanaka, 1964). This might be due to the enzymatic action of the fungus on the cuticle of the insect as demonstrated by Samsinakova et al. (1971) in the cuticle of greater wax moth larvae, G. mellonella, while assaying the enzymatic activity of the fungus, B. bassiana. Tanaka (1964) has stated that no external symptoms appeared in the early stages of white muscardine in silkworm larvae, perhaps, it may be due to the fact that only trained eye could recognize the changed colour in the early stages of the disease as it would be very feable.

The sluggishness and loss of appetite in the larvae observed here, found to be common in all mycoses of the insects (Madelin, 1963) and silkworms infected with B. bassiana (Mukerji,1912; Dasgupta, 1950; Anonymous, 1956; Tanaka, 1964) and M. anisopliae (Glaser, 1926). This has been observed in European corn borer larvae, Ostrinia nubilalis (L.) infected with B. bassiana (Lefebvre, 1934), maize stem borer, Chilo zonellus Swinhoe larvae (Mathur et al., 1966), grubs of alfalfa weevil, Hypera postica (Gyll.) infected with B. bassiana densa (Subba Rao et al., 1968) and grubs of coconut beetle, Oryctes rhinoceros L. infected with M. anisopliae (Nirula et al., 1955).

Decreased irritability of the diseased larvae might be due to the loss of sensation of the skin as a result of the fungal invasion. Similar reports have been made in silkworms infected with M. anisopliae (Glaser, 1926), P. nubilalis larvae (Lefebvre, 1934), grubs of H. postica (Subba Rao et al., 1968) infected with B. bassiana and grubs and beetles of O. rhinoceros infected with M. anisopliae (Nirula et al., 1955). Paralysis and loss of elasticity of the larval body of the silkworm have been reported earlier (Mukerji, 1912; Anonymous, 1956; Krishnaswami et al., 1973).

The presence of black or dark-brown spots on the integument of majority of the diseased larvae (Fig. 1), agrees with the reports of Kurisu (1962) and Krishnaswami et al. (1973). There were a few larvae which did not show any black spots, similar to the report of Katsumata (1932) that no black spots were observed in spite of morbidity of the infected larvae. Aoki and Yanase (1970) have stated that the infection of B. mori by B. bassiana may or may not produce the black spots. They concluded that the occurence of these black spots was due to less disturbance and non-occurence of them was due to the drastic disturbance caused by the fungus in the phenol oxidase activity of the integument, thus the infected larvae in latter case died before they could show any black spots. The occurence of black spots in V instar larvae was noticed during 4.0 to 6.5 days Similarly Aoki and Yanase (1970) recorded slight dark patches on the integument of silkworm larvae inoculated with B. bassiana spores within 4-5 days. The number of spots on integument was generally five, although upto 22 per larva was recorded. No such report is available. The per cent larvae showing black spots increased as the instar of the larvae increased (Table III).

Oily substance oozing out from the black spots (Fig. 1) observed in severely infected worms. This agrees with the reports of Kurisu (1962), Aoki and Yanase (1970) and Krishnaswami et al. (1973). Aoki and Yanase (1970) further stated that the oily oozing was due to the severity of the disease as they could not observe it in mildy infected larvae.

The cessation of feeding, frequent diahrroea with agony and vomiting of gastric juice by the larvae approaching death observed here is similar to the early reports of Tanaka (1964) and Krishnaswami et al. (1973). The external deformations reported by some workers in other insects were not observed here. Generally, the death of the infected larvae occurred anywhere from 4 to 11 days, which confirms the reports made by earlier workers.

The cadaver turned red or pink in colour (Fig. 2a) after 4 to 12 days after inoculation. This symptom was first recorded in the silkworm larvae by Balsamo (1835) and later by several workers (Mukerji, 1912; Steinhaus, 1949; Tanaka, 1964; Krishnaswami et al., 1973). It was observed in P. nubilalis (Lefebvre, 1934) and wharf piling borer beetles (Benham and Miranda, 1953) infected with the fungus, B. bassiana. Of the 66.6 per cent of the red coloured larvae the colouration may be due to the red pigments, 'bassianin' secreted by the fungus (El Basyuouni et al., 1968). But in the remaining cadavers the colouration was due to the secondary invasion of the red pigmented bacterium, S. marcescens. This agrees with the reports made by Siemaszko (1937) and Siemaszko and Jaworsky (1939). The absence of red colouration in a few cadavers might be due to the fact that the fungus might not produce enough red pigments or no pigments.

The stiffening of the body, as observed here, has been previously reported (Mukerji, 1912; Dasgupta, 1950; Tanaka, 1964; Krishnaswami et al., 1973). The white efforescence, started first over the intersegmental regions and later all over the body and the 'lime covered stick' like appearance of the corpses, has been already reported by almost every worker who studied this disease.

Coremia, perithecia, sclerotia and white crystals were not observed. Coremia (Petch, 1931), perithecia and sclerotia (Paillot, 1929) and white crystals (Verson, according to Steinhaus, 1949) have been reported. However, these structures were not found to be of regular occurence among the hypomycetous fungi (Madelin, 1963).

The diseased larvae took more time to spin the cocoon compared to the time taken by the healthy larvae. Some diseased larvae died during spinning and resulted in one fourth and half spun cocoons (Fig. 4). Similar observations were made by Krishnaswami et al. (1973).

Cocoon:

There was no difference between the cocoons fully spun by diseased larvae and the cocoons spun by healthy larvae. But the former ones sounded like dried cocoons, with dead pupae, when shaken. Except Arbousset (1893) who found the infected cocoons brighter and slightly transparent, no other report on the symptoms of the diseased cocoon is available. White efforescence on the pupae dead inside the cocoons (Fig. 5) and decrease in weight of the diseased cocoons were noticed, similar to the reports of Arbousset (1893) and Krishnaswami et al. (1973). It did not come out to the surface of the cocoons because of the impermeability of the shell. More than 95 per cent of the pupae formed out of the diseased larvae did not survive. As a result of this, no moths emerged from the cocoons. The emerged moths from these cocoons were with crinkled wings (Fig. 7) and died soon. Mukerji (1912) and Arbousset (1893) have made a few similar observations.

The pupae, removed from healthy cocoons and inoculated with the fungal spores, became soft and uneasy within seven days. No such reports in silkworm are available. However, Nirula et al. (1955) observed such symptoms in O. rhinoceros pupae infected with M. anisopliae. The diseased pupae died in about 9-11 days and became more soft at first, hardened later and then covered with white efforescence. These symptoms were observed by Krishnaswami _et_ _al_. (1973).

Moth.

The infected moths were sluggish and evinced less interest in copulation. This observation agrees with the report of Arbousset (1893). There was no external symptoms of the disease before death and also no report is available. The infected males even though sluggish did not decline to copulate healthy females, when provided, and were able to mate successfuly. The moths died in 2-3 days after inoculation which is close to the observations made by Arbousset (1893). In case of codling moth, _Laspeyresia_ _pomonella_ moths died in 4-6 days of inoculation (Primak, 1967). The fungus was found to be more concentrated in the ovary in females compared to the testis of males. This probably explains the early death of the females compared to the males.

All the dead moths showed red colouration. The hardening, white efforescence especially on intersegmental regions of the abdomen and drying of the corpses were observed. These symptoms have been reported earlier (Arbousset, 1893; Krishnaswami _et_ _al_., 1973). The white efforescence all over cadaver was not found unless the dead moths were kept in high humid conditions, which shows the importance of RH in the development of the fungus (Walstad _et_ _al_., 1970).

Route of infection

Among the three methods of inoculation of the fungal spores, namely, intra-haemocoelic injection, topical and oral inoculation to the silkworm larvae, the former one gave the highest mean larval mortality (47.75) followed by second and third methods (Table IV). This might be mainly because of the fungus was directly injected into the haemolymph. Whereas in latter two methods the integument acted as a barrier which slowed down the entry of the pathogen into the haemolymph. In case of intra-haemocoelic injection the fungus saved a period of atleast 20 hours required to penetrate the cuticle of the larvae, thus it caused early death. The fungus penetrated the integument of the silkworm larvae in 24-48 hr (Steinhaus, 1949) and the larvae of gypsy moth, Porthetria dispar (L.) in 24 hr (Wasti and Hartman, 1975).

The topical application gave the second highest mean larval mortality (43.25). The route of infection in this method obviously was through the integument. The Hypomycetous fungi entering the body through the cuticle has been well established in insects including silkworm (Steinhaus, 1949; Madelin, 1963, 1966; McCoy, 1974).

The mean mortality of the larvae fed with spores along with the food registered only 27.75, was not highly significant from the mortality of the larvae injected with distilled water. However, this revealed that the fungus could infect the silkworm larvae Per Os. This has been showed in silkworm larvae (Anonymous, 1956; Gabriel, 1959), corn borer larvae, P. nubilalis (Metalnikov and Toumanoff, 1928; Lefebvre, 1934), grubs of Melolontha melolontha (Hurpin and Vago, 1958), termites (Bao and Yendol, 1971), honey bee, Apis mellifera (Burnside, 1930) and larvae of imported fire ant, Solenopsis ritcheri (Broome et al., 1976). Ferreira (1943) and York (1958) have observed the germination of B. bassiana spores in the digestive tract which were ingested along with food by P. nubilalis larvae indicating that the ingested spores can germinate and infect the host insect.

There was no significant difference, among the mortality of the larvae treated with spores by different methods and also among the controls (Table IV).

Life cycle of the fungus, B. bassiana

The life cycle of the fungus could not be studied on the eggs of the silkworm as the fungus did not sporulate on the surface. It is not reported in insect eggs also.

The period of the life cycle of the fungus on an average was 6.1 days in larvae (V instar), the maximum and minimum being 10.5 and 4.5 days, respectively. This agrees with the report of Steinhaus (1949), that the fungus sporulated on the larval cadaver of silkworm in 24-48 hr and the time required to kill the larvae varied from 3 to 10 days (Mukerji, 1912; Krishnaswami et al., 1973; Chitra et al., 1975). Sikura and Simchock (1972) also reported the fungus reached its full developmental cycle within 5-6 days in the wood moth caterpillar, Zeuzera pyrina L. and Urs et al.(1965) found it completely covering the larvae of cabbage semilooper, Plusia sp. in 3 days.

The fungus completed its life cycle in 3.5 days on Sabouraud's maltose agar, which is almost a half of the period of its life cycle in silkworm larvae (6.1 days). This may be mainly due to the fact that the availability of oxygen required for the development of the fungus was unlimited at the surface of the culture media compared to the limited availability inside the larval body. Samsinakova and Hrabetova (1969) found the fungus utilising more oxygen in its initial stages and decreased with the age of the fungus in submerged culture. Phagocytosis may be another factor contributing to the delayed life cycle of the fungus in silkworm (Steinhaus, 1949), atleast in the initial stages.

The shape of the spores differed depending upon the site of sporulation. Spores produced inside the body were cylindrical compared to globular conidia on the surface of the cadaver. Kurisu (1962) has made similar report and Samsinakova (1966) has reported formation of similar spores (blastospores) in submerged culture within three days of inoculation.

The fungus to complete its life cycle took, on an average, 10.5 days in the silkworm pupae. This prolonged period might be due to the resisance of the pupa (Sussman, 1952; Koidsumi, 1957) against the invasion of the fungus. In view of the present day practice of cutting open the cocoons for early moth emergence, this finding has an important bearing in silkworm rearing.

The fungus completed its life cycle in 2.0 to 3.5 days (on an average 2.65 days) in moths of the silkworm. Arbousset (1893) and Tanaka (1964) have also reported early death of the moths due to the disease compared to larvae and pupae, as they were more susceptible.

The life cycle of the fungus was completed in, on an average, 3.69, 3.00, 4.50, 6.00 and 6.13 days in I, II, III, IV and V instars, respectively (Table V). This variation in different instars may be due to comparatively bigger size of the V instar larvae and phagocytosis of fungal spores in initial stages of the disease by the blood cells of the silkworm. Arbousset (1893) found the fungus taking a week to complete its life cycle in the V instar larvae. Chitra _et al_. (1975) reported that the death of the larvae occurred within 2-3 days in younger larvae and 3-5 days in full grown larvae. If the period of efforescence of 1-2 days (Steinhaus, 1949) is added to the period in which the larval mortality occurred, the life cycle of the fungus would be 3-5 days in younger larvae and 4-7 days in older larvae, which supports the present findings.

The fungus took 3.88 to 11.56 days to complete its life cycle during various seasons of the year 1976-77, in V instar larvae. Similar period of 4-10 days is recorded earlier (Anonymous, 1956). The fungus took largest period in November-December 1976 and shortest period in May-June 1977 (Table VI). There was no significant difference between October-November and November-December 1976 and so also between May-June and June-July 1977 with respect of fungal life cycle period. January-February 1977 significantly differed from other seasons at five per cent level. This variation was mainly due to the fluctuations in temperature (23-28°C) at a relatively constant RH. Temperature and RH are well known factors influencing the fungal development and sporulation (Walstad _et_ _al_., 1970).

The fungus took on an average 7.13 and 5.25 days in Pure Mysore and HS_6 races, respectively to complete its life cycle (Table VII). These races differed significantly from others and there was no significant difference among other races. Perhaps Pure Mysore and HS_6 withstand the disease for longer period, probably due to their varied genetical characters.

Susceptibility of different stages of the silkworm, _B. mori_

The silkworm eggs were less susceptible to the fungus, _B. bassiana_ compared to other stages (Table VIII). Freshly laid eggs showed higher mortality compared to the aged eggs.

There is no report of infection of silkworm eggs by B. bassiana. However, Bartlett and Lefebvre (1934) found B. bassiana infecting P. nubilalis eggs in moist conditions, eggs of Epilachna varivestis Mulsant and Musca domestica L. (Dresner, 1949). Primak (1967) found that 16.67 per cent eggs failing to hatch when B. bassiana conidia were sprayed onto the codling moth eggs. Majchrowicz and Yendol (1973) found no infection of P. dispar eggs in the field sprayed with B. bassiana spores and no recovery of the viable spores on the egg surface. This may be due to adverse environmental conditions in the field.

Larvae was found to be the most susceptible stage. Larval mortality upto 100 per cent was obtained in some of the experiments. Although all the instars were susceptible to the disease, the V instar was found to be most susceptible as compared to other instars (Table IX). The per cent larval mortality in different instars differed significantly from one another at one per cent level. It was found decreasing from I to II instars which may be attributed to the duration of II instar (2 days). It was a short period left for the fungus to penetrate, develop and sporulate. Zacharuk (1974) found, in his studies on the elaterid beetles infected with M. anisopliae, that the germ tube of the spore could not penetrate as the old cuticle gave way to the new one in the process of moulting, thus preventing the fungus reaching the body. It could be presumed that similar process takes place in II instar. From III instar onwards the per cent larval mortality increased (Table IX), which agrees with the early findings (Arbousset, 1893; Mukerji, 1912; Dasgupta, 1950).

Different larval instars were inoculated with spores to observe the mortality and in the succeeding stages. It followed the sequence as in the above experiment in relation of larval mortality (Table X). In all the instars the inoculated instars showed highest mortality and the succeeding instar or stage showed increased mortality from the next instar or stage of inoculation, except in the II instar where the inoculated instar showed low mortality compared to the next instar. The number of healthy cocoons formed was more in I instar and it decreased as the number of instars increased (Table X). No such reports are available.

The larvae treated before IV moult showed low mortality compared to the larvae treated after IV moult. The V instar larvae inoculated on different days showed decreasing mortality as the age advanced (Table XI). There was no appreciable difference in the per cent mortality of larvae inoculated at moulting and the day before and after IV moult. The mortality of the V instar larvae inoculated on different days did not differ significantly. The V instar larva was found to be most susceptible on its first day, as it registered 71.5 per cent mortality. Kawakami (1975) has also reported that 1-2 days old V instar larvae were most susceptible to <u>A. flavus</u> and it decreased with lapse of time.

Effect of different concentrations of B. bassiana spores on the silkworm, B. mori

The larval mortality showed direct relationship with concentration of spore suspesnsion as it increased with increase in spore concentration in all the instars (Fig. 8). Pristavko and Yanishevskaya (1971) reported 15, 35 and 90 per cent mortality of the larvae of *L. pomonella* inoculated with 10^2, 10^3 and 10^4 spores of *B. bassiana* per larva. Fargues (1972) has shown similar relationship of *B. bassiana* spores to *M. melolontha* grubs under laboratory conditions.

The median lethal concentration (LC_{50}) of spores required to I instar was highest (1598) and it decreased to 435 spores for V instar (Table XII). But Kawakami (1975) found that LC_{50} of *A. flavus* spores increased with increase in instars. However, *A. flavus* is found to be less pathogenic to insects including *B. mori* and more saprophytic in nature compared to *B. bassiana*.

The median lethal time (LT_{50}) was minimum in V instar (8.27 days) and highest in III instar larvae (13.57 days) at 10^3 spores/ml suspension. It was found to increase with decrease in spore concentration used to inoculate different larval instars (Table XIV). However the variation in LT_{50} in different instars (Table XIII) could not be correctly assessed as there is no such information available on silkworm. This is shown in Fig. 9. The LT_{50} of IV (Fig.10) and V (Fig. 11) instar increased with decrease in spore concentration.

The cocoon was the most resistant stage, as it showed no permeability to the fungus invasion, even after it was dipped in the spore suspension for ten minutes. However, Bartlett and Lefebvre (1934) did not get the moths emerged from *P. nubilalis* cocoons dipped in spore suspension, might be due to the permeability of the cocoon.

The pupae from the healthy cocoons inoculated with fungal spores showed 40.85 per cent mortality. This agrees with the findings of Koidsumi (1957) and Tanada (1955) that pupa was less susceptible due to its waxy cuticular layers. As the age of the pupae advanced they became less susceptible. This agrees with Sussman (1952) who found the pupae of *Cercopia* silkworm infected with *A. flavus*. The pupae inside the cocoons spun by the infected larvae died in majority of the cases and thus resulted in no moth emergence. Bartlett and Lefebvre (1934) and Krishnaswami *et al.* (1973) have observed no moth emergence from cocoons formed by the infected larvae of *P. nubilalis* and *B. mori*, respectively.

The mortality of the pupae, formed by the larvae inoculated with *B. bassiana* spores in different instars, showed no relationship with the spore concentration used. But it seemed to have some relationship with lapse of time between the inoculation of the larvae with spores and their pupation (Table XV). Number of the healthy cocoons spun by the survived larvae increased as the *B. bassiana* spore concentration decreased (TableXVI

Next to the larval stage, moths were the second most susceptible to the fungus. But Arbousset (1893) found that moths were more susceptible than larval stage. Krishnaswami et al. (1973) have reported the mortality of the moths similar to the present findings. On the contrary Mukerji (1912), Picard (1913) and Steinhaus and Bell (1953) have reported the non-susceptibility of the moths of silkworm, B. mori Phthorimoea operculella and Sitotroga cerealella, respectively, to B. bassiana infection. However, L. pomenella (Primak, 1967) and P. nubilalis (Smith and York, 1960) moths have been reported to be susceptible to the fungus, B. bassiana.

The virgin moths were less susceptible than the mated ones. Similar report is available on chinch bug, Blissus leucopteres (Billings and Glenn, 1911). The mated females were comparatively less susceptible than females which already laid their eggs. Arbousset (1893) and Billings and Glenn (1911) made similar observations in silkworm and chinch bug, respectively. The inoculated moths laid less number of eggs (101.5 to 256.3 on an average) compared to healthy ones (513.7 on an average). The reports on reduced fecundity due to mycoses are available (Billings and Glenn, 1911; Pascalet, 1939; Schaerffenberg, 1964).

Effect of B. bassiana on different silkworm races

Among eleven races of the silkworm screened against white muscardine disease to test the degree of the susceptibility Pure Mysore was found to be comparatively less susceptible. J_{122} race was most susceptible at all the concentrations of

B. bassiana spores tested. The other races of the silkworm came in between these two races with respect of larval mortality (Table XVII, Fig. 12). The percent pupal mortality of 0.5 in races HM II, NB_4D_2 and the maximum of 3.25 in C. Nichi was recorded.

The number of healthy cocoons spun by the survived larvae of different races of the silkworm was highest in Pure Mysore at 10^3 and 10^6 spores/ml suspension and C. Nichi at 10^8 spores/ml suspension used to inoculate and J_{122} showed least number of healthy cocoons and no cocoons were obtained at 10^8 spores/ml suspension used to inoculate its larvae. C. Nichi, commonly believed to be more susceptible to the diseases of the silkworm found, superior to many other races as it gave higher number of cocoons. Devaiah (1973) also found C. Nichi better over other indigenous races in respect of the pebrine disease. The other races came in between Pure Mysore and J_{122} (Table XVII, Fig. 12).

The race, Pure Mysore was better than F_1s of the crosses, having it as their female parent. The cross Nandi also showed no improvement over the mid-parent value. However, this would not agree with the report of Aruga (1957) that the parents were more susceptible than their F_1 hybrids to the polyhedral diseases.

SUMMARY

VI. SUMMARY

The studies on several aspects of the white muscardine disease of the silkworm, Bombyx mori Linnaeus caused by the fungus, Beauveria bassiana (Balsamo) Vuillemin are summarized here.

The Isolates of the fungus, B. bassiana from the infected silkworms, obtained from Bangalore, Kolar, Mandya and Mysore showed variation in culture and infectivity to the silkworms. The Isolate B was found more infective to the silkworms compared to the other Isolates. Conidia were globose in shape and measured from 1.5 to 2.9 μ in diameter. One mg spore material contained about 10^8 spores. Of the seven media used for culturing the fungus, Sabouraud's maltose agar with one per cent yeast was found to be the best suited.

The symptoms of the white muscardine disease of the silkworm were studied at all the stages of the silkworm. Infected eggs turned pale or pinkish in colour, looked partially filled and the chorion shrunk. The infected larvae showed fading of usual shiny nature of their skin and became sluggish, lost irritability and elasticity, stopped feeding, exhibited black or brown spots with oily oozing, became soft followed by diarrhoea with agony and ultimately died. The larval cadavers turned red in colour, later covered with white efforescence and dried into mummies resembling 'lime covered sticks'. Infected pupae became soft, uneasy and later died. These cadavers were also covered by the white efforescence. The infected moths were sluggish, and showed less interest in copulation. Their cadavers also showed red colouration and white efforescence.

The route of infection of the fungus in the silkworm larvae was mainly the cuticle and the digestive tract.

The fungus to complete its life cycle from spore to spore took 6.10, 10.50 and 2.65 days in the larva, pupa and moth, respectively and 3.69, 3.00, 4.50, 6.00 and 6.13 days in I, II, III, IV and V instar of the silkworm larvae, respectively. The period of life cycle of the fungus was found varying from 3.88 to 11.56 days in various rearing seasons of the year 1976-77. It was highest in November-December 1976 and lowest May-June 1977 rearing seasons. It varied from 5.25 to 7.13 days in V instar larvae of different races of the silkworm.

All the stages of silkworm were found susceptible to the disease. Egg, larva, pupa and moth stages registered 5.7, 99.9, 40.9 and 78.1 per cent mortality, respectively, due to the disease. The V instar was the most susceptible as it showed 99.55 per cent mortality compared to 45.92, 36.25, 60.20 and 88.67 per cent mortality in I, II, III and IV instar, respectively. The susceptibility of the V instar larvae was found to be maximum on its first two days and it declined later.

The mortality of the larvae increased as the concentration of the spores of the fungus, *B. bassiana* used to inoculate them increased. The median lethal concentration of *B. bassiana* spores to the newly moulted larvae of I, II, III, IV and V instar was 1598.0, 1410.0, 1171.0, 601.6 and 435.0, respectively. The median lethal time was 11.89, 11.15, 13.57, 13.18 and 8.27 days in I, II, III, IV and V instar larvae, respectively, at 10^3 spores/ml suspension. It increased with decrease in the spore concentration used in IV and V instar larvae. The mortality of the pupae formed by the infected larvae showed no definite relationship with the spore concentration.

All the eleven races of the silkworm, screened against the disease were susceptible to the fungus as they showed mortality at all the three concentrations of *B. bassiana* spores. The Pure Mysore race showed lesser larval mortality and thus resulted in higher number of healthy cocoon formation as compared to other races. The Japanese race, C. Nichi excelled over other races in respect of number of cocoons formed.

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*Originals not seen.

APPENDICES

APPENDIX I

Effect of different concentrations of *Beauveria bassiana* spores on different larval instars of the silkworm, *Bombyx mori* L.

	I instar								
No. of spores/ ml suspension	10^9	10^8	10^7	10^6	10^5	10^4	10^3	10^2	0
*No. of larvae died on									
III day	44	29	26	16	17	6	4	0	0
IV day	115	94	81	73	58	38	14	6	9
V day	233	243	226	235	134	130	109	55	6
VI day	65	55	60	55	46	36	42	24	1
VII day	12	10	8	14	18	15	13	20	0
VIII day	120	58	54	50	52	23	30	13	1
IX day	14	11	23	12	20	37	13	11	0
X day	19	26	46	72	72	62	70	12	4
XI day	23	58	25	29	40	63	65	13	2
**XII day	119	111	107	98	96	119	96	75	2
Total:	764	695	656	654	553	529	456	229	25
No. of pupae died	6	4	17	10	3	3	10	0	0
No. of healthy cocoons formed	30	101	127	136	244	268	334	571	775

	II instar								
No. of spores/ ml suspension	10^9	10^8	10^7	10^6	10^5	10^4	10^3	10^2	0
*No. of larvae died on									
III day	216	158	160	121	101	41	31	8	0
IV day	52	52	59	47	50	154	112	59	6
V day	5	22	16	13	26	24	26	11	1
VI day	15	19	15	16	9	12	15	13	2
VII day	22	24	56	13	7	20	8	6	0
VIII day	368	355	301	298	197	99	100	53	2
IX day	9	13	6	48	36	64	58	9	2
X day	16	8	5	14	11	15	17	10	2
XI day	8	11	7	14	19	10	9	6	1
**XII day	58	93	69	85	98	92	85	65	7
Total:	769	755	694	669	554	531	461	240	23
No. of pupae died	11	5	3	4	6	5	6	4	0
No. of healthy cocoons formed	20	40	103	127	240	264	333	556	777

	III instar								
No. of spores/ ml suspension	10^9	10^8	10^7	10^6	10^5	10^4	10^3	10^2	0
*No. of larvae died on									
III day	72	51	57	35	21	5	1	0	0
IV day	167	126	108	82	76	62	47	16	2
V day	273	247	240	184	147	166	159	88	3
VI day	127	136	96	91	92	64	48	6	8
VII day	9	23	15	18	19	21	11	2	0
VIII day	8	14	9	3	16	9	8	3	0
IX day	8	17	9	29	1	2	5	5	2
X day	14	16	32	44	8	6	28	3	0
XI day	14	74	78	78	48	50	47	26	0
**XII day	108	59	79	117	132	148	134	93	6
Total:	800	763	723	681	560	533	488	242	21
No. of pupae died	0	12	9	9	5	5	5	4	0
No. of healthy cocoons formed	0	25	68	110	235	262	307	554	779

	IV instar								
No. of spores/ ml suspension	10^9	10^8	10^7	10^6	10^5	10^4	10^3	10^2	0
*No. of larvae died on									
III day	38	28	14	8	3	0	1	0	0
IV day	77	52	49	48	43	20	7	1	1
V day	141	130	74	77	58	58	61	33	0
VI day	246	204	176	123	112	115	103	55	3
VII day	26	41	66	64	57	56	41	45	0
VIII day	44	48	56	46	55	18	30	28	0
IX day	51	53	55	51	30	14	20	10	0
X day	49	57	63	40	33	36	8	3	0
XI day	61	90	101	101	68	72	68	40	4
**XII day	67	87	86	145	135	150	168	56	4
Total:	800	790	740	703	594	539	507	271	12
No. of pupae died	0	1	4	4	2	1	1	9	0
No. of healthy cocoons formed	0	9	56	93	204	260	292	520	788

	V instar								
No. of spores/ ml suspension	10^9	10^8	10^7	10^6	10^5	10^4	10^3	10^2	0
*No. of larvae died on									
III day	36	32	9	3	0	0	0	0	0
IV day	87	84	64	64	45	39	34	23	0
V day	252	226	214	207	138	69	60	53	0
VI day	215	158	193	114	179	113	102	102	0
VII day	175	144	100	103	113	100	121	47	0
VIII day	21	67	78	78	75	77	81	32	3
IX day	2	39	68	59	39	57	63	4	2
X day	5	18	2	49	3	51	34	35	0
XI day	5	20	7	24	4	19	7	15	0
**XII day	2	12	10	13	2	9	4	9	0
Total:	800	800	745	714	598	534	506	320	5
No. of pupae died	0	0	2	3	0	8	4	1	0
No. of healthy cocoons formed	0	0	53	83	202	258	290	479	795

* Based on four replications each with 200 larvae.

** Onwards upto cocoon formation.

APPENDIX II

Provisional probits* for per cent mortality of different instar **silkworm larvae inoculated** with different concentrations of <u>Beauveria</u> <u>bassiana</u> spores.

Sl. No.	Numbers of spores per ml suspension	I instar		II instar		III instar		IV instar		V instar	
		1	2	1	2	1	2	1	2	1	2
1.	10^9	95.50	6.53	96.13	6.87	100.00	7.07	100.00	7.17	100.00	7.25
2.	10^8	86.88	6.25	94.38	6.54	95.38	6.72	98.75	6.81	100.00	6.90
3.	10^7	82.00	5.97	86.75	6.21	90.38	6.37	92.50	6.46	93.13	6.55
4.	10^6	81.75	5.69	83.63	5.88	85.13	6.02	87.88	6.11	89.25	6.20
5.	10^5	69.13	5.41	69.25	5.55	70.00	5.67	74.25	5.76	74.75	5.84
6.	10^4	66.13	5.13	66.38	5.23	66.63	5.32	67.38	5.41	66.75	5.49
7.	10^3	57.00	4.95	57.63	4.90	61.00	4.98	63.38	5.06	63.25	5.14
8.	10^2	28.63	4.57	30.00	4.56	30.25	4.63	33.88	4.71	40.00	4.79
9.	0	3.13	-	2.87	-	2.63	-	1.50	-	0.63	-

* From Fig. 8

1 Per cent mortality

2 Provisional probit (Y)