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Research Article

# ISOLATION AND MORPHOLOGICAL CHARACTERIZATION OF A FUNGAL ISOLATE OBTAINED FROM MUSCARDINE DISEASED MUGA SILKWORM *ANTHERAEA ASSAMENSIS* HELFER (LEPIDOPTERA: *SATURNIIDAE*)

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**Abstract-** Muga silkworm, *Antheraea assamensis* Helfer is an economically important sericigenous insect and produces unique golden colour silk. Muga silkworm is endemic to North Eastern States of India. The silkworm is polyphagus, multivoltine and reared outdoor mainly on leaves of Som (*Persea bombycina*) and Soalu (*Litsea monopetala*) trees. Since Muga silkworms are reared on outdoor conditions, diseases and pests are the major constraints for enhancing production and productivity. Muscardine or mycosis is a deadly fungal disease occurred mostly in winter crops and thereby causing death of considerable number of Muga silkworm larvae which ultimately affects the cocoon production. Nevertheless, information on characterization and lifecycle of the fungal isolate obtained from muscardien diseased Muga silkworm is obscure. In the present study muscardine (mycosis) infected dead mummified Muga silkworm larvae have been collected and fungus has been isolated (isolate MSFP1). The fungal isolate was cultivated in different media. Asexual reproductive stages of the fungi have been identified as microconidia and blastospores. Different developmental stages of conidiogenous cells and blastospores were documented. Based on the conventional morphological characters and microscopic observations, the fungal isolate MSFP1 has been putatively identified as *Beauveria* sp. under 'Hypocreales' order and 'Cordycipitaceae' family.

**Keywords**- *Muga silkworm, Antheraea assamensis Helfer, Fungal pathogen, Muscardine disease, Beauveria sp., Asexual stages*

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## Introduction

North East India enjoys the unique distinction of producing all the four major varieties of commercially exploited silks *viz*. Eri, Muga, Tasar and Mulberry. Of particular importance is the Muga silkworm *Antheraea assamensis* Helfer which is endemic to North East region of India and produces the glittering unique golden colour silk. Practicing Muga culture is a part of the rich tradition and cultural heritage of North East India people. Muga culture plays a significant role in sustainable rural livelihood generation and poverty alleviation. The production of Muga raw silk in India during 2017-18 was estimated to be 192 MT (http://texmin.nic.in/). On the basis of environmental condition and cocoon quality entire muga silkworm crops are divided in to three crops *i.e*. commercial , seed and pre-seed crop and all crops are equally playing a key role in muga silk industry. This silkworm is polyphagous and multivoltine (06 crops year-1) in nature and feeds primarily on 'Som' (*Persea bombycina*) and 'Soalu' (*Litsea monopetala*). Due to outdoor nature of muga silkworm rearing, outbreak of various diseases *viz*., pebrine, flacherie, muscardine and grasserie are the major constrains encountered in Muga silk industry [1]. Even after providing quality foliage and suitable environmental conditions, in every crop around 14-40% crop loss normally occurred due to incidence of such diseases [1]. Among the Muga silkworm diseases, muscardine or mycosis caused by fungal pathogens occurred mostly in winter crops (December, January, and February) and thereby causing death of considerable number of Muga larvae which ultimately affects the cocoon production. Considerable crop loss of around 60-90% may occur, If Jarua pre-seed crop (December-January) is affected by muscardine which hampers the availability of seed for subsequent Chatua seed crop (February-March). Earlier three different species of entomopathogenic fungi such as *Beauveria bassiana*, *Aspergillus niger* and *Fusarium moniliforme* were isolated from diseased Muga silkworms [2, 3]. White muscardine caused by *Beauveria bassiana* was found to be a most common fungal infection in Bombyx mori L during rainy and winter seasons in India. Out of the total disease occurrence in mulberry silkworm, 10 - 40 % crop loss is accounted for muscardine disease as reported by earlier worker [4]. Green muscardine caused by *Metarhizium anisopliae* in Bombyx mori L was also studied [5]. The information pertained on morphological, molecular and etiological aspects of muscardine disease in mulberry silkworm and its causative pathogen has been well established. Nevertheless, such type of information is limited in case of Muga silkworm *Antheraea assamensis* Helfer except few studies [2, 3]. In order to increase Muga cocoon production and productivity, management of muscardine disease is inevitable which largely depends on characterization of the fungal pathogen and host-pathogen interaction. In the present study morphological and microscopic observations of a fungal pathogen isolated from white muscardine infected mummified Muga silkworm larvae during late Kothia crop (October -November) were documented. Different reproductive stages of the fungal isolate MSFP1were reported.

## Materials and Methods

Muscardine infected mummified dead Muga silkworm cadavers were collected from rearing field during late Kothia crop (November 2018). The infected Muga silkworm larvae were early 5$^{th}$ instar larva reared upon Soalu plantation (*Litsea monopetela*). Isolation of entomopathogenic fungus was carried out as per the standard protocols [6, 7]. In brief, growing fungus from mummified Muga silkworm (*Antheraea assamensis* Helfer) larva was transferred to Sabouraud dextrose agar with 1% yeast extract (SDAY) (HiMedia Laboratories Pvt. Ltd, Mumbai)

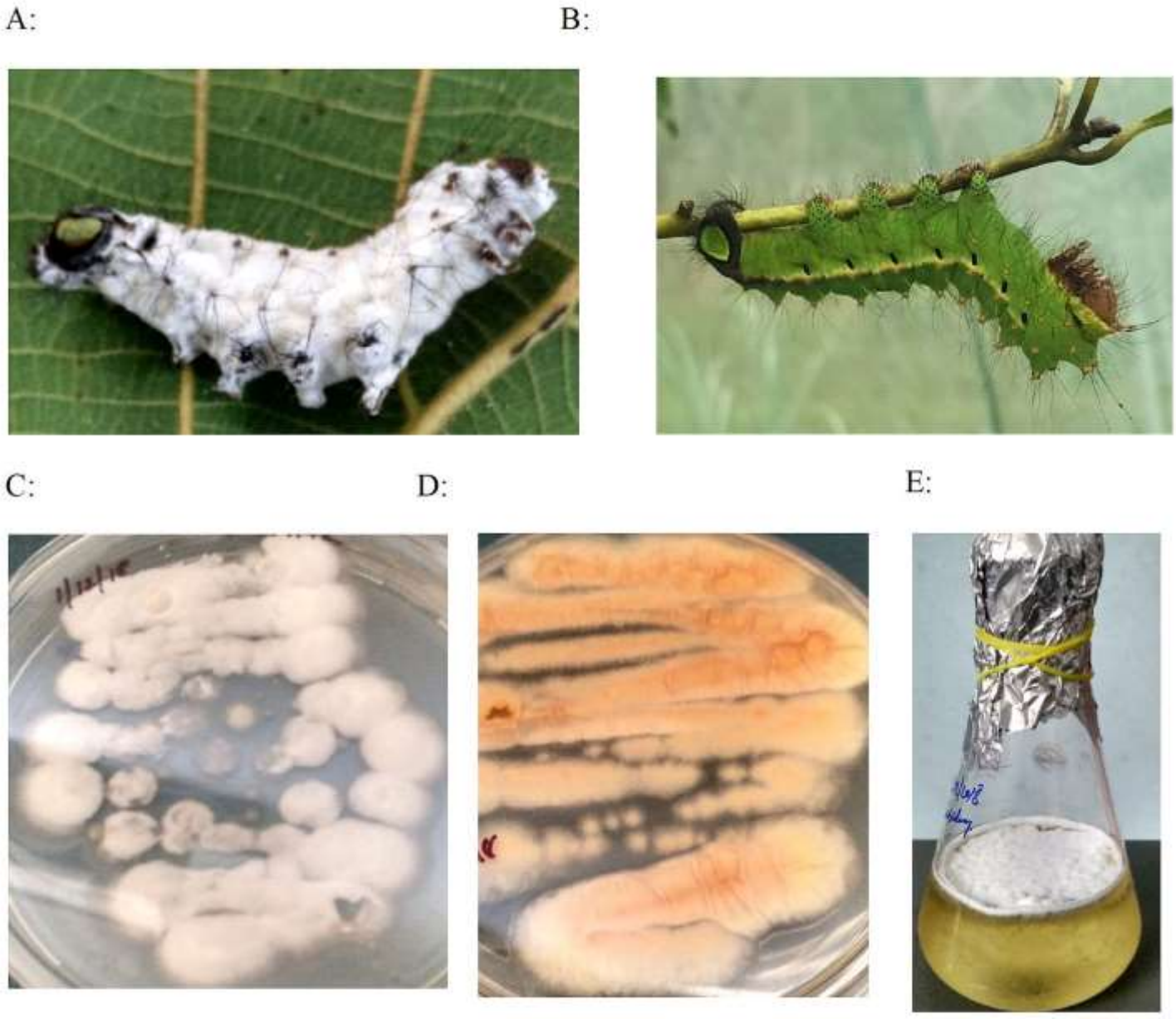



Fig-1 Muscardine infected mummified dead cadaver of 5th instar Muga silkworm larva (A) and healthy Muga silkworm larva (B). White coloured aerial conidia on Muga silkworm integument are prominent in Muscardine disease. Culture plate of fungal isolate MSFP1, surface view (C) and bottom view (D). E: Fungal culture on SDY broth

supplemented with antibiotics (streptomycin sulfate 350 mg/l$^{-1}$, Chloramphenicol 100 mg l$^{-1}$ (HiMedia Laboratories Pvt. Ltd, Mumbai). Addition of antibiotics to the SDAY medium is to prevent any bacterial colonization and promote selective growth of fungal mycelium. Plates were incubated in total darkness at 25°c and RH 70% for 10 days. Developing colonies were sub-cultured on new SDAY agar plates. From these cultures, monosporic culture (Isolate AA1) was obtained by plating a serially diluted conidia suspension of one colony forming unit (CFU). The growing mycelia and aerial conidia were microscopically observed with Lactophenol–Cotton blue staining and examined under x400 and x1000 (oil immersion) magnifications on a Nikon Eclipse E600 microscope (Nikon, Japan). Aerial conidia from monosporic cultures were harvested by flooding the plate with sterile double distilled water. Conidial suspensions were later filtered through a single layer of cotton pad and final spore concentrations were determined by direct counting using a haemocytometer. Blastospores of entomopathogenic fungi were produced in Sabouraud dextrose+1% yeast extract liquid broth cultures (SDY) using conidia harvested from plates to a final concentration of $10^5$ cells ml$^{-1}$ as the inoculums [8]. Cultures were grown for 4 days at 20°C. Then the cultures were filtered through glass wool to remove mycelia, and the concentration of blastospores was determined by direct counting. Blastospores were microscopically observed with Lactophenol–Cotton blue staining and examined under x400 and x1000 (oil immersion) magnifications on a phase contrast microscope. Morphological identification of the fungus was done as per standard procedure [9]. Aerial conidia from the monosporic isolates were stored at –20°C in 15% glycerol.

**Results and Discussion**

Mycosis infected Muga silkworm larvae shows loss of appetite, vomiting of gut juice, ceases to move, loses elasticity of integument, appearance of moist shiny specks on the dorsal side of the integument. When compared to healthy Muga larvae, diseased dead Muga larval body initially soft, become rubbery, turn to harden and finally mummify (approx. 48 hrs to 72 hrs of death) due to surface coverage of millions of white powdery aerial conidia [Fig-1A and B]. In general, mummified larval stage was considered to be highly contagious and responsible for horizontal transmission of the infection in the rearing field. The Muga cadaver covered with white powdery mycelium and produces millions of conidia except the parts of the head capsule, true legs and anal plate [Fig-1A]. We anticipate that the fungus penetrates only the thinner, non-sclerotised areas of the cuticle, like joints, between segments or the mouth parts.

After 10 days of the incubation, the fungal isolate MSFP1 colonies were characterized to be bright white and the bottom of the colony is pale yellowish in colour [Fig-1C and D]. The colonies produced many dry, hydrophobic powdery conidia in distinctive white spore balls. Each spore ball is composed of a cluster of mono-nucleated conidiogenous cells. A white colour fungal mat in SDY broth was noticed after 4 days of incubation [Fig-1 1E]. Microscopic observation with Lacto phenol staining indicated that fungal hyphae was thin and found to be branched, septate, and hyaline [Fig-2].

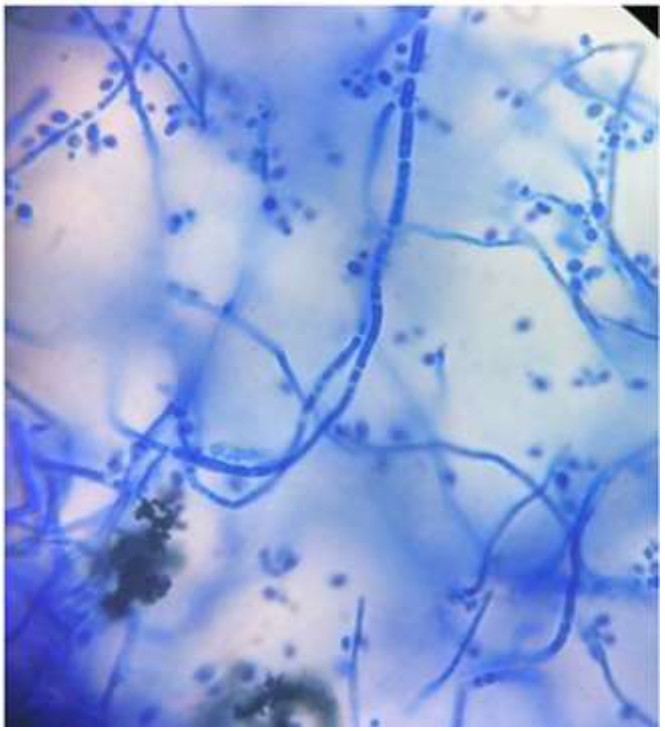
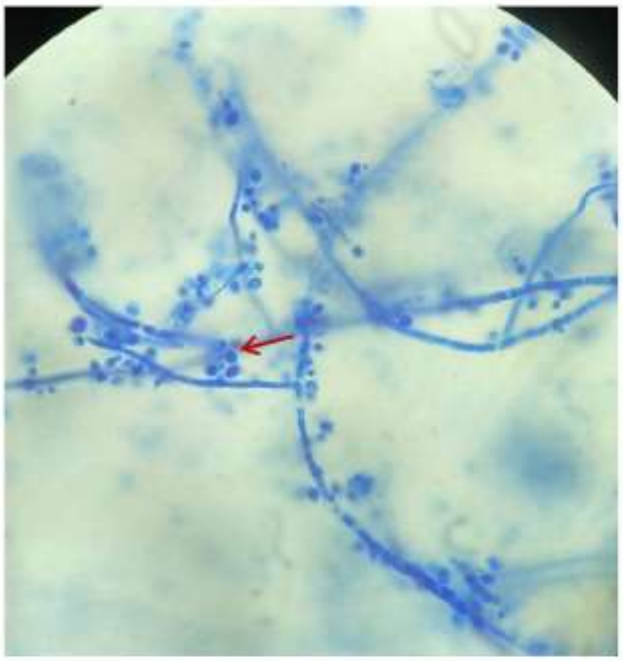


Fig-2 Lactophenol cotton blue staining of growing mycelia of fungal isolate MSFP1 on SDAY agar pale (x 1000 magnification). Sepatate hyphe was notced.

Fig-3 Lactophenol cotton blue staining of fungal isolate MSFP1 obtained from Muga silkworm. The photograph was taken under light microscope with x 450 magnification. Arrows indicates the aerial conidia on hyaline mycelia

The conidiogenous cells are short and ovoid, terminate in a narrow apical extension called a rachis [Fig-3; Fig-4A and B]. Sympodial arrangement of conidia was observed [Fig-4A and B). Aerial conidia were appeared to be single celled, hyaline, globose to broadly ellipsoidal [Fig-4A]. Phase contrast microscopic images revealed different developmental stages of microconidia of fungal isolate MSFP1 [Fig-5A]. Four prominent asexual conidial stages (I to IV) have been shown. Stage I represents formation of flask-shaped, rachiform, proliferating conidiogenous cell. Stage II represents maturation of conidia and formation of new conidiogenous cell in acropetal arrangement where youngest conidia located at tip and the oldest conidia located at base. Stage III consists of extension of conidiophores and showing sympodial development of single-celled conidia. Stage IV shown extension of conidiophores on a geniculate or zig-zag rachis emanating from a flask-shaped conidiophore. Further, enlarged view (x1000) of microconidia on a geniculate or zig-zag rachis documented [Fig-5B). The rachis elongates after each conidium is produced, resulting in a long zig-zag extension [Fig-5A and B). The microconidia are formed in clusters, like cotton balls. The morphological and microscopic observations of fungal isolate MSFP1documented in this study are in accordance with fungal genera taxonomically placed under the order 'Hypocreales' and family 'Cordycipitaceae' family.

A:



B:

Fig-4 Sympodial arrangement of conidiogenous cells on septate hyaline mycelia (A). Mono-nucleated conidiogenous cells (Shown with arrow) short and ovoid, terminate in a narrow apical extension called a rachis. Enlarged view of aerial conidia and rachis (B). The photograph was taken under light microscope with x 1000 magnification.

Further, according to Humber, 1997 [9], the colony characters, morphological and microscopic observations along with pathological attributes of the fungal isolate are found to be to *Beauveria* sp. Similar kinds of characteristics were noticed with *Beauveria bassiana* infected Bombyx mori L by previous works [5, 10-12]. Earlier three different species of entomopathogenic fungi such as *Beauveria bassiana*, *Aspergillus niger* and *Fusarium moniliforme* were isolated from diseased Muga silkworms [2, 3]. Nevertheless, the present study was mainly focused on the documentation of morphological and reproductive characters of fungus isolated from white muscardine disease of Muga silkworm. This information would be useful for better understanding the life cycle of the *Beauveria* sp. in Muga ecosystem. Most species of entomopathogenic fungi were taxonomically placed in to either Entomophthoromycota or the *Ascomycota* divisions. For a long time *Beauveria* had placed within the fungal division Deuteromycetes, because of its unknown sexual stage.

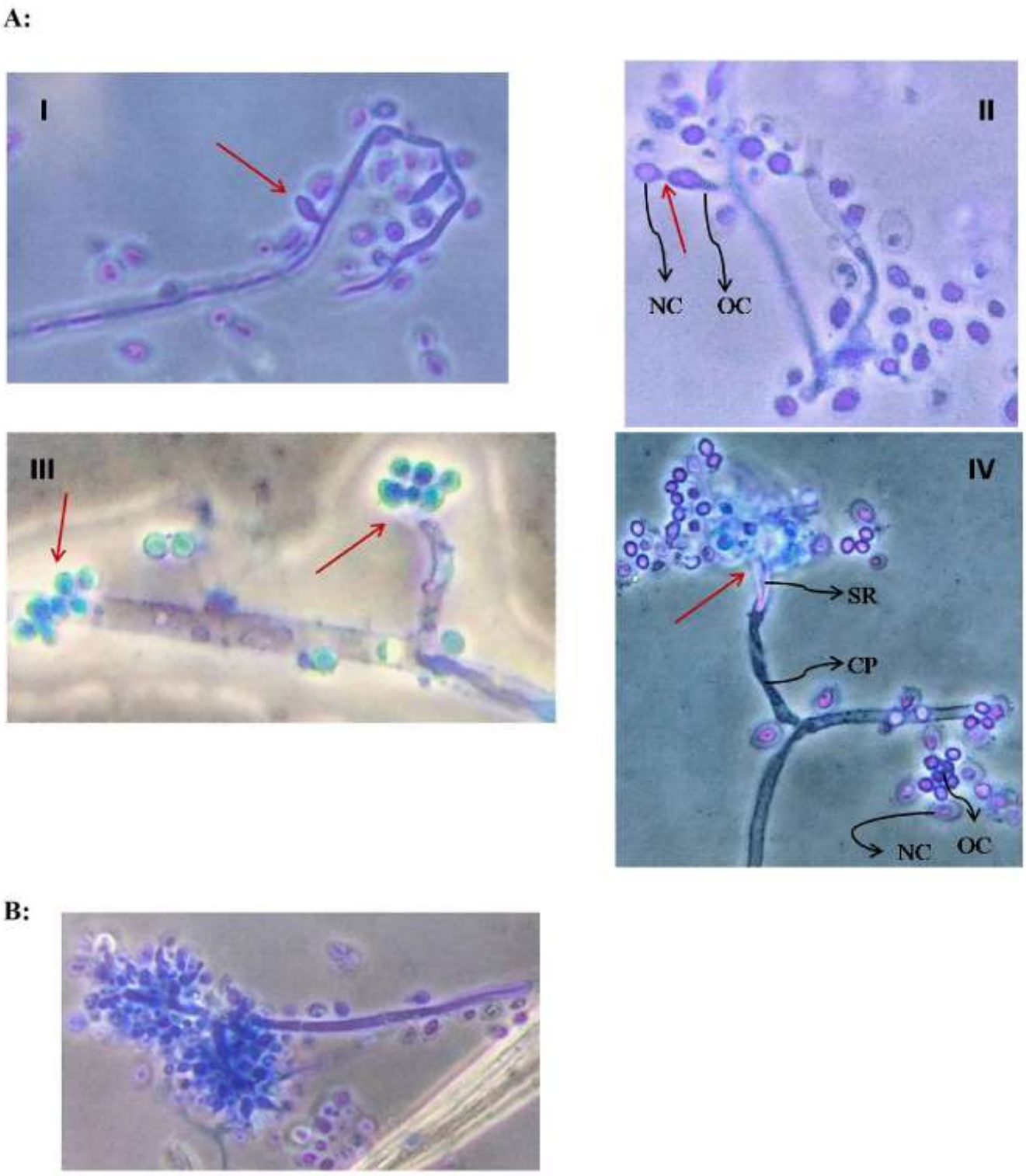



Fig-5 Phase contrast microscopic images of different developmental stages of aerial conidia in fungal isolate MSFP1. Four prominent stages (I to IV) havebeen shown (A). Stage I: Formation of flask-shaped, rachiform, proliferating conidiogenous cell; II: Maturation of conidia and formation of new conidiogenous cell in acropetal arrangement where youngest conidia located at tip. III: Extension of conidiophores showing sympodial development of single-celled conidia; IV: Extension of conidiophores on a geniculate or zig-zag rachis emanating from a flask-shaped conidiophore. OC: Old conidia; NC: Newly formed conidia; SR: Sympodial rachis; CP: Matured and extended conidiophores. Enlarged view of microconidia on a geniculate or zig-zag rachis.

However, recent molecular techniques (DNA-sequence based) have allied *Beauveria* within the Ascomycete genus Cordyceps and resulted in its reassignment [13]. In this study, formation of typical oval to irregular shaped blastospores of MSFP1 in nutrient rich SDAY broth was observed after the 4 days incubation. Phage contrast microscopic (x 1000) images indicated that the size of the blastospores were relatively bigger than aerial conidial structures of *Beauveria* sp. MSFP1 [Fig-6A and B]. An interesting phenomenon presumed to be fission type asexual reproduction of blastospores was observed and same was documented [Fig-7]. We anticipate six different prominent stages (I to VI) associated with blastospores fission [Fig-7]. Blastospores production of *Beauveria* sp in nutrient rich medium was earlier reported, however their shape was found to be cylindrical [14]. It has been emphasized that aerial conidia, vegetative blastospores and submerged conidia, of *Beauveria* sp., display different morphological, biochemical and virulence properties [14].

Single-cell infectious propagules of *Beauveria bassiana* are known to be blastospores or aerial conidia which infect insects via attachment to the host integument. It was found that all life stages of the fungus (hyphae, aerial conidia, submerged conidia, single-cell blastospores) appeared to be infectious [8]. However, the extent to which these fungal forms infect insects in nature is largely unknown and attempts are being made to exploit these properties in pest targeting or the enhancement of virulence. Mono-nucleate cells of *Beauveria* spp. display distinct morphological, biochemical and pathological characteristics [8].

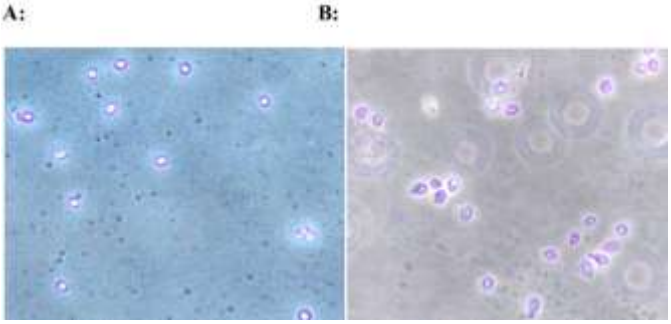



Fig-6 Phage contrast microscopic images of asexual reproductive stages of fungal isolate MSFP1. A: Micro conidia; B: Blastospores.

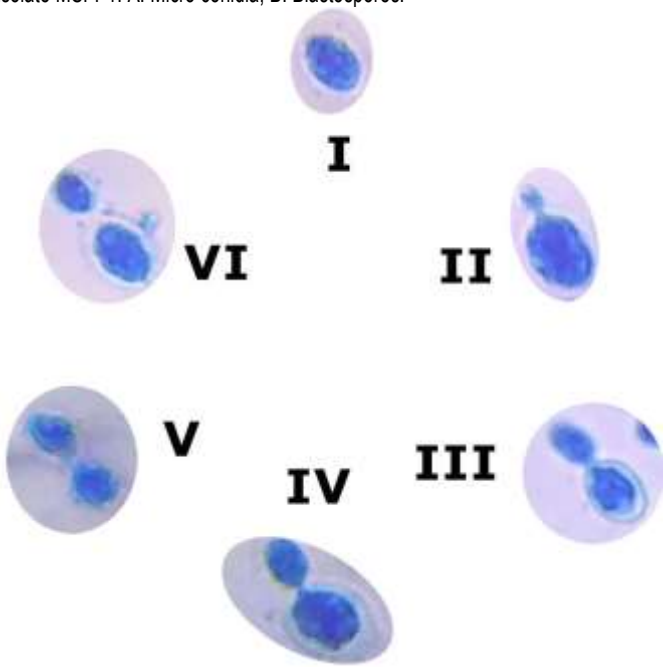



Fig-7 Different asexual reproductive stages (I to VI) of blastospores of fungal isolate MSFP1 in Sabouraud dextrose broth. Blastospore production was maximum at 96 hrs. Blastospores are multiplied by yeast like fission. Six distinct prominent stages (I to VI) of blastospores fission have shown in the figure. The photograph was taken under light microscope with x 1000 magnification.

Asexually produced mitosporic aerial conidia are considered to be the main dispersal and infectious structures and are resistant to a greater extent to various abiotic/biotic stresses. Aerial conidia are hydrophobic in nature and selectively bind to hydrophobic surface of insect epicuticle [15]. Hydrophobic interactions between *B. bassiana* conidia and insect epicuticle are facilitated by pathogen derived proteins known as hydrophobins *viz*. Hyd1 and Hyd2. These proteins have been characterized [16, 17]. Monoclonal antibodies synthesized against the tagged versions of Hyd1 and Hyd2 revealed that Hyd1 was localized on the surface of aerial and submerged conidia. Hydrophobin Hyd2 was found only on the surface of aerial conidia and at the base of the germinating conidia. Nevertheless, neither of these proteins found on blastospores indicating a specialized unknown infectious mechanism of blastospores. Contact angle measurements using polar and apolar test liquids revealed that cell surfaces of mono nuclear aerial conidia were hydrophobic, whereas those of blastospores and submerged conidia were hydrophilic in nature [8]. As a non-motile organism, *Beauveria* sp. target Muga silkworm through a phenomenon of passive transmission of asexual conidia with airborne, water dispersed, contaminated leafs, soil, rearing appliances and physical contact with infected insects. Cross-transmission of *Beauveria* sp. in Muga rearing field is not yet established. However, considering the broad host range of pathogen, it could be anticipated that muscardine may be cross-transmitted to Muga silkworm through other insect visitors in rearing field [18]. Therefore, *Beauveria* sp. infection can be viewed as an opportunistic event mediated by aerial conidia that happen to find themselves on Muga silkworm cuticle. Pathogenic and parasitic mechanisms of *Beauveria bassiana* were attributed to expression of virulent genes and concomitant secretion of degradative/digestive enzymes and toxic molecules. Production of enzymes and toxic substances may intern facilitates fast assimilation of nutrients, rapid growth and inhibition/resistance to host defense mechanisms. Many of these processes are intrinsically nonspecific and lead to saprophytic growth. In addition to its saprophytic growth, *B. bassiana* also tend form stable endophytic associations with plants rhizosphere and on the phylloplane [19-21]. Thorough knowledge on entomopathogenic fungi is considerably important to control and/or mange onset of fungal infections in Muga rearing filed. Fungal isolate MSFP1 was identified as *Beauveria* sp., on the basis of colony characters, pathological attributes, morphological and microscopic observations. Molecular studies Although traditional identification of species in the genus *Beauveria* is principally based on conidial morphology, molecular phylogenetics has revealed that the genus includes many cryptic diversifications that makes species identification a challenge [13, 19, 22-24]. One study indicated that entomopathogenic fungus *Beauveria* comprises 12 distinct cryptic species such as *B. amorpha, B. asiatica, B. australis, B. bassiana, B. brongniartii, B. caledonica, B. kipukae, B. malawiensis, B. pseudobassiana, B. sungii, B. varroae, B. vermiconia* (23). Low temperature and high humidity plays a big role for the occurrence of muscardine disease in B. mori L as the fungus grows well in relatively high humidity 90 to 95% and low temperature < 25ºC [25]. Similarly, muscardine incidence in Muga silkworm rearing is frequently noticed in Jarua (December-January) and Chatua (February-March) winter crops. Especially heavy fog and winter showers during the aforesaid seasons increases the humidity and there by aggravates the situation. It was reported that continuous cloudy weather and foggy climate (for 3-4 days), medium temperature with high humidity (>80%) are the predisposing factors for white muscardine disease in Muga silkworm rearing [3]. Although Climate change may recognize as a major threat to the biodiversity and integrity of ecosystems, the rise in global atmospheric temperature may also has certain positive effects in Muga silk industry. The maximum and minimum temperature recorded in December 2012 at CMERT&TI, CSB, Lahdoigarh were 27.6ºC and 5.5ºC respectively. While maximum and minimum relative humidity in December 2012 were 92% and 72% respectively. These climatic conditions (low temperature <6.0°C and high humidity) are considered to be predisposing factors for higher incidence of deadly muscardine disease consecutively noticed in Jarua crop of 2012, 2013 and 2104. However, during December 2016-2017, the incidence of muscardine disease in Jarua crop was not reported in lower and upper Assam regions. So far, it is presumed that this development can be attributed to the increased temperature and lower humidity in December 2015 and 2016. The maximum and minimum temperatures in December 2016 at CMERT&TI, Lahdoigarh were 29.1ºC and 10.0ºC respectively and the maximum and minimum relative humidity in December 2016 were 82% and 44% respectively. These changes in climatic condition over a period of time are a good sign for production and productivity of Jarua pre-seed crop as revealed by institutional "Forecasting and forewarning calendar for Muga silk worm diseases" (www.cmerti.res.in). However, the Institute has observed unexpected incidence of muscardine disease in Chatuaand early Jethua crops of 2016 & 2017 during which heavy rain fall was occurred. These results indicate a link between contrasting climate conditions and muscardine incidence. We presume that there may be likely occurrence of muscardine disease in Chatua and Jethua crops if the rain fall occurs or other predisposing factors prevail. The rainy winter seasons with high humidity are congenial for the spread of white muscardine disease. Hence, it is important to resort to appropriate management practices to prevent the outbreak of the disease at the field level.

**Application of research**: This study would be useful for better understanding of morphological and reproductive stages of *Beauveria* sp and their incidence with respect to climatic conditions during Muga silkworm rearing.

**Research Category**: Silkworm Pathology

**Abbreviations**: SDAY: Sabouraud dextrose yeast extract agar; SDY: Sabouraud dextrose yeast extract broth

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**Research project name or number**: Research station trials

**Author Contributions**: SG designed the study, collected samples, performed experiments, interpreted data, and wrote the paper. KM, KD, CM and DR performed experiments and reviewed the manuscript. DR guided the work.

**Author statement**: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrollment

**Conflict of Interest**: None declared

**Sample Collection**: Muga silkworm cadavers were collected from rearing field during late Kothia crop (November 2018).

**Ethical approval**: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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