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Isolation and characterization of white muscardine fungi *Beauveria bassiana* (Bals.) Vuill. - A causative of mulberry silkworm

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Abstract
The present paper presents research on the isolation of pathogen, pathogenicity test and standardization of culture media, susceptible stage of infection. The isolated fungal pathogen appeared as initially white in color and later turn into yellowish in color and the spores were spherical, oval and non septate. The pathogenicity test proved that artificially inoculated *B. bassiana* in healthy silk worms showed typical symptoms of white muscardine. The maximum growth *B. bassiana* has supported by SDA medium by recording the mycelial growth of 85.3 mm. The highly susceptible stage of silkworm for muscardine disease percent were the larvae inoculated more than 12 hours before moulting and six hours after moulting by recording 100 per cent mortality.

Keywords: Silkworm, *Beauveria bassiana*, sericulture, white muscardine fungi

1. Introduction
Mulberry sericulture is mainly practiced in five states *viz*, Karnataka, Andhra Pradesh, West Bengal, Tamil Nadu and Jammu and Kashmir which jointly account for about 97% of the total mulberry silk production in the country [3]. The quality and productivity of bivoltine silk are better than that of multivoltine silk [13]. In Tamil Nadu, mulberry is cultivated in the districts of Krishnagiri, Dharmapuri, Coimbatore, Thiruppur, Theni, Salem, Erode, Tirunelveli, Tirchy, *etc*. Mulberry area in Tamil Nadu is 41,482 acres with raw silk production of 1418 MT [1]. Tamil Nadu continues to occupy the fore-front position in the country in bivoltine silk production. Silkworms are susceptible to a number of diseases caused by different infectious agents such as fungal, bacterial, viral and protozoan diseases. It is the main factor seriously affecting the cocoon production [6]. Among the insect pathogens, fungi constitute the largest group with more than 700 species causing mycosis in insects [19]. Fungal disease in insect is called muscardines or mycoses. Nearly a dozen species of fungi cause infections in silkworm of which most of the infections are caused by the members of the genera are *Beauveria* and *Metarhizium*. They are found throughout the world and are most contagious [17]. Infection of silkworm by *Beauveria bassiana* causing white muscardine is the first entomopathogenic fungus to be observed as early as 1835 by Bassi. In his honour, Balsamo described and named the fungus *Botrytis bassiana*. It is Vuillemin who created the genus *Beauveria* and selected *bassiana* as the type species in 1912. It is known to infect 750 arthropod species [4]. This disease wiped out the entire sericulture industry in Italy and France during 1920-1925 [15]. In Japan and India a loss of 10-40 per cent of total loss due to diseases is accounted for white muscardine [21]. During the early stages of infection the color of the silkworm changes from creamy white in colour. The worms become inactive, lose appetite and motionless. The body elasticity is lost and the larvae become soft to touch. The death occurs in about 2-10 days [14]. Prevention of silkworm diseases has become one of the most important aspects in the success of commercial sericulture. Objective of the present study Isolation and characterization of pathogen associated with white muscardine fungi in silkworm using cultural practices *viz*., morphological character and physiological growth requirements on culture media and proving the pathogenicity.

2. Materials and Methods
2.1 Isolation and Maintenance of white muscardine fungi in silkworm
White muscardine infected silkworms collected based on visual examination from the Department of Sericulture, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu state

(India) during September, 2013. Collected samples were first microscopically examined in order to confirm the presence of the white muscardine pathogen using the binocular research microscope (at 10x). After confirming for the presence of *B. bassiana* spores they were isolated in the laminar flow chamber under aseptic conditions following standard isolation method [9]. The infected larvae showing typical symptoms were cut into small bits and surface sterilized with 70% ethanol rinsed thrice with water were transferred to sterile petriplates containing Sauboured Dextrose Agar (SDA) under aseptic conditions. The inoculated petri plates were incubated under sterilized bell jars at room temperature (27±1ºC) and observed at regular intervals. A loop full of fungal culture developed on the bits on SDA plates were taken on a glass slide and observed under the microscope for the presence of conidia and conidiophores. After confirming the spores of the cultures, they were purified by single spore isolation technique. The fungus was sub cultured on Sauboured Dextrose Agar (SDA) and allowed to grow for seven days at 27±1ºC and preserved at 4ºC, sub cultured under aseptic conditions periodically. The pathogen was closely observed for the cultural, morphological and spore characters in the culture media and also observed under microscope.

2.2 Pathogenicity test for *B. bassiana*

The pathogenicity of the fungus was verified by artificially inoculating on first day of fifth instar healthy silkworm under *in vitro* condition. The observations were taken. The pathogen from the infected silkworm was re-isolated and compared with primary culture.

2.3 Methods of inoculation for *B. bassiana*

Twenty five larvae of a popular bivoltine silkworm hybrid (CSR2×CSR4) were used for the experiments. *B. bassiana* was cultured in petriplates using Sabouraud's dextrose agar. Conidia were harvested by brushing the surface of three week-old culture into a 500 ml glass beaker containing 50 ml sterile distilled water using a sterile camel hair brush.

A drop of tween-20 was added to the beaker containing distilled water to keep the conidia dispersed. The conidial suspension was prepared by mixing the solution using a magnetic stirrer for 5 minutes and its concentration was determined based on counts made with an improved Neubauer haemocytometer. The required concentration of *B. bassiana* inoculum (1×10^5 conidia/ml) was prepared by suitably diluting the stock inoculum with steriled distilled water [20]. Newly ecdysed fourth instar larvae (out of third moult) were counted 100 each and topically inoculated by dipping them in *B. bassiana* inoculum suspension of 1×10^5 conidia/ml concentration. High relative humidity (95±5 % RH) was provided by keeping wet foam pads in the rearing trays and a temperature of 25±1ºC was maintained in the rearing room [18]. White muscardine incidence was recorded for ten days post inoculation. LT50 and LT90 values were calculated for each feed schedule by probit analysis.

2.4 Standardization of culture media for *B. bassiana*

Pure cultures of *B. bassiana* were obtained from Tamil Nadu Agricultural University, Coimbatore. Isolates of *B. bassiana* were maintained at the Department of Sericulture, Tamil Nadu Agricultural University, Coimbatore during 2013-14. The fungal isolates were maintained in the standard media and incubated for 10 days at 25ºC. Dishes showing good fungal growth were selected for the experimental inoculation. Semi synthetic media used were Sabourauds dextrose agar with yeast (SDA+Y) [22], Sabourauds maltose agar with yeast (SMA+Y), Potato dextrose agar (PDA), Rose bengal agar (RBA). The composition contained 20g/l of media along with Potato extract (200 g/l) and agar (20g/l). Observations on radial growth, biomass and spore count were recorded as described [10]. All the statistical analysis were done using IRRISTAT [8].

3. Results

3.1 Isolation and Pathogeneicity of *B. bassiana*

The *B. bassiana* isolates at first appeared as white colour, later becoming yellowish and powdery type. Spore structure were characterized *viz.,* spherical or oval and non septate (Plate 1a and 1b). The pathogenicity of *B. bassiana* was proved by artificially inoculated the pathogen to healthy silkworm under *in vitro* conditions showed the typical symptoms of white muscardine disease and also re-isolation of pathogen from diseased silkworms were similar to that of primary culture.

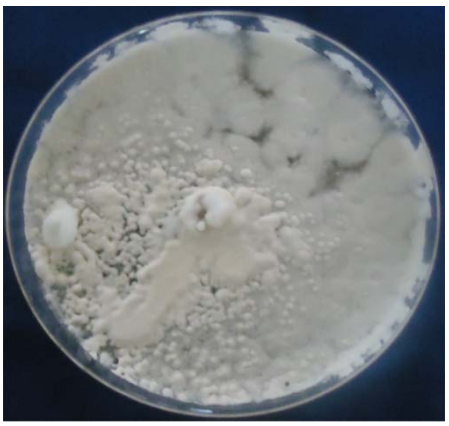

Plate 1a: Culture Plate of B. bassiana

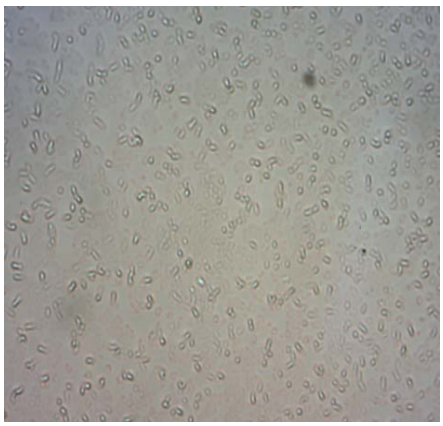

Plate 1b: Conidia of B. bassiana

3.2 Standardization of culture media for *B. bassiana*

The experiment on standardization of culture media for the isolation of *B. bassiana* with different media *viz.,* Sabourauds dextrose agar with yeast (SDA+Y), Sabourauds maltose agar with yeast (SMA+Y), Potato dextrose agar (PDA), Rose bengal agar (RBA). The results showed that the media Sabourauds dextrose agar with yeast (SDA+Y) recorded the highest mycelial growth (85.3 mm) in seventh day next followed by Sabourauds Maltose agar with yeast Medium (SMA+ Y) registered the mycelial growth of 78.6 mm. (Table, 1; Fig, 1).

Table 1: Standardization the culture media for *B. bassiana*

S. No	Semi synthetic medium	Mean mycelial growth (mm)*		
		3rd day after inoculation	5th day after inoculation	7th day after inoculation
1.	Sabouraud's Dextrose Agar with Yeast Medium (SDA + Y)	30.5	51.4	85.3
2.	Sabouraud's Maltose Agar with yeast Medium (SMA + Y)	29.4	50.7	78.6
3.	Potato Dextrose Agar Medium (PDA)	28.4	44.6	76.6
4.	Rose Bengal Agar Medium (RBA)	28.7	41.8	72.5
	CD (P = 0.05)	0.2174	0.3480	0.3351
	SED	0.0943	0.1509	0.1453

Values are mean of three replications
Means followed by a same letter are not significantly different at the 5% level by DMRT.

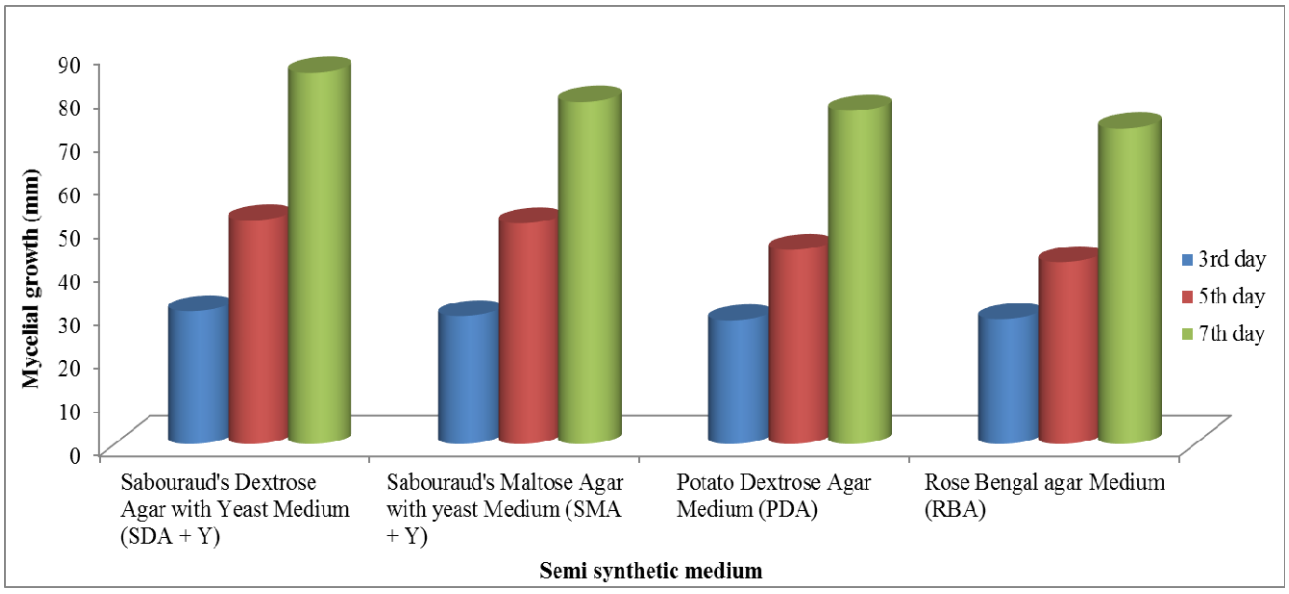

Fig 1: Standardization the culture media for *B. bassiana*

3.3 Susceptibility stage of infection due to *B. bassiana*

The results of the experiment on influence of moulting of silkworm on
B. bassiana infection showed that the susceptibility of *B. bassiana* indicated that the larval mortality 100 per cent were recorded in the larvae was inoculated more than 12 hrs before moulting and more than 6 hrs after moulting respectively (Table, 3).

Table 3: Susceptibility stage of infection due to *B. bassiana*

Time of inoculation	Per cent mortality due to white muscardine disease during different instars			
	I Instars	II Instars	III Instars	IV Instars
18 HBM	100.00±0.00 (90.00)	100.00±0.00 (90.00)	100.00±0.00 (90.00)	100.00±0.00 (90.00)
12 HBM	100.00±0.00 (90.00)	100.00±0.00 (90.00)	100.00±0.00 (90.00)	100.00±0.00 (90.00)
6 HBM	100.00±0.00 (90.00)	100.00±0.00 (90.00)	64.67±2.52 (48.16)	34.30±2.52 (35.06)
Moulting	37.33±4.50 (36.86)	30.00±1.50 (33.25)	9.40±1.25 (18.00)	1.00±1.00 (4.62)
6 HAM	100.00±0.00 (90.00)	100.00±0.00 (90.00)	100.00±0.00 (90.00)	100.00±0.00 (90.00)
12 HAM	100.00±0.00 (90.00)	100.00±0.00 (90.00)	100.00±0.00 (90.00)	100.00±0.00 (90.00)
18 HAM	100.00±0.00 (90.00)	100.00±0.00 (90.00)	100.00±0.00 (90.00)	100.00±0.00 (90.00)
SED	0.9767	0.6189	0.6914	0.7715
CD (P= 0.5)	2.0951	1.3275	1.4832	1.6549

HBM: Hours before moulting
HAM: Hours after moulting
Figures in parenthesis are angular transformed values.

4. Discussion

Silk yield by silkworms are greatly affected by various diseases. White muscardine disease due to *B. bassiana* causes a cocoon yield loss upto 30 per cent almost throughout the year [11]. The white muscardine fungi is a notoriety causative on several beneficial and hazardous insect orders *viz*., orders Lepidoptera, Homoptera, Coleoptera, Hemiptera and Diptera, which were distributed throughout the world [2]. *B. bassiana* cultures were isolated from the disease infected cadavers in SDA medium. The *B. bassiana* isolates were characterized based on the colony colour and spore structure and identified as *B. bassiana*. *B. bassiana* produced velvety to powdery type colony which looks white in the beginning but later becoming yellowish in colour, spores are spherical or oval and non-septate. Similar observations were earlier observed by different authors [5,7,12] in which they found that the *in vitro* colonies of *B. bassiana* appear velvety to powder, at first white then turn yellowish. Artificial inoculation of white muscardine fungus on healthy silkworms showed the similar symptoms as that of original one. The fungus re-isolated from

artificially inoculated dead cadaver also similar to that of primary culture. Standardization of culture media for *B. bassiana* with different media was tested. Among the culture media, Sabourauds dextrose agar with yeast (SDA+Y) recorded the highest mycelial growth (85.3 mm). These results were in accordance with the earlier findings [22]. The larvae inoculated with *B. bassiana* more than 12 hours before moulting and more than 6 hours after moulting recorded the highest (100%) mortality (sucestible stage to *B. bassiana* infection). The mortality and fecundity of silkworm was accordance with earlier findings [16, 23] in which they found complete annihilate of cadaver.

5. Conclusion

The pathogen *B. bassiana* was isolated and identified based on the cultural and morphological characters *viz.,* first white colour, later becoming yellowish and powdery type. Spore structure was spherical or oval and non septate and suitable medium for growth and sporulation was standardized. Also the susceptibility stages and inoculation methods for proving the infectious nature was optimized in the present study.

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