Cellular abnormalities induced by *Trichoderma* spp. during *in vitro* interaction and control of white muscardine *(Beauveria bassiana)* and green muscardine *(Metarhizium anisopliae)* disease of silkworm *Bombyx mori*

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ABSTRACT

The silkworm is the larva of the domesticated silk moth, *Bombyx mori*. It is an economically important insect, being a primary producer of silk in sericulture industry. Among the silkworm diseases, white muscardine and green muscardine caused by *Beauveria bassiana* and *Metarhizium anisopliae* respectively possess a major threat to silk cocoon production. White muscardine is more common during rainy and winter seasons whereas green muscardine has its profound effect during hot and humid spells. Both these fungi *Beauveria bassiana* and *Metarhizium anisopliae* can be used as biopesticides to control a number of pests such as termites, whiteflies, and many other insects from larvae to adult stages. In this paper *in vitro* biological control of *B. bassiana* and *M. anisopliae* and cellular abnormalities induced by the application of two strains (T12 and T13) of *Trichoderma viride*, *Trichoderma harzianum* and *Trichoderma* spp. were studied, where *T. viride* T 12 (80.52%) provides maximum *in vitro* control of *B. bassiana* followed by *T. harzianum* (71.88%), *Trichoderma* spp. (68.16%) and *T. viride* T13 (62.89%). Against *M. anisopliae, T. harzianum* provides maximum *in vitro* control (68.02%), followed by *T. viride* T13 (64.68%), *T. viride* T12 (59.47%) and *Trichoderma* sp. (57.98%). During the interaction of pathogens and biocontrol agents hyphal coiling, granulation, distortion, vacuolation and bulging were recorded.

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INTRODUCTION

Silk production is the ultimate goal of sericulture and mulberry silkworm *B. mori*, which is an economically important primary producer of tradable silk, a class of fibre of excellence, grace and luster (Nataraju *et al.*, 2005). India has unique distinction of being the only country in the world bestowed by nature with all the four known species of silkworm viz., Mulberry, Eri, Muga and Tasar. Mulberry sericulture is practiced in major parts of southern India. Mulberry silkworm, *Bombyx mori* L. is affected by number of diseases. In 1807 Augastino Bassi first discovered white muscardine disease of silkworm and later the causal organism was named after his name *Beauveria bassiana*. The disease induced serious economic loss in France and Italy (https;//www.britanica.com). In 1950, Dasgupta reported major silkworm diseases caused by Grasserie (virus), Flacherie (bacteria), Muscardine (fungi) and Pebrine (protozoan /microspordian). Among the fungal diseases of silkworm, white muscardine and green muscardine possess a major threat to silk cocoon production during rainy and winter seasons as these two seasons are congenial for the spread of these diseases (Sengupta *et al.*, 1990, Lu-Yun-Lian, 1991). Both white muscardine caused by *B. bassiana* and green muscardine caused by *M. anisopliae* are well known entomopathogenic fungi. Although *B. bassiana* and *M. anisopliae* are frequently used for biological control of many aphids, insects like *Leucinodes orbanalis* (Pal and Ghosh, 2014) as they have the potentiality to

infect and kill the aphids from larval to adult stage, induce up to 50% loss to the death of silkworm larvae (*B. mori*) (Jayaramaiah and Kuberappa, 1987) which ultimately leads to enormous loss in sericulture industry and silk production. Generally to combat these diseases chemicals are frequently used, but prolonged use of these chemicals are environmentally hazardous and toxic. A variety of alternative approaches have been adopted among which use of biocontrol agents like *Trichoderma* spp. has been widely implemented for the management of fungal diseases in crop plants. The application of *Trichoderma* spp. has proved fruitful against many soil borne and foliar pathogens. Recently PUSA has developed formulations from *T. viride, T. harzianum, T. viriens* for soil application which alone or in combination has proved to be highly efficient against several diseases of vegetables, cereals, pulse, spices, fruits etc. (Sharma *et al*., 2014; Pandian *et al.,* 2016).

Therefore, the main objective of this work is *in vitro* control of the pathogens by the antagonistic efficacy of different biocontrol agents like *T. viride* T12, *T. viride* T 13, *T. harzianum* and *Trichoderma* spp. and to record the cellular abnormalities like hyphal coiling, granulation, distortion, vacuolatation, bulging between hyphal interaction of pathogens and biocontrol agents.

MATERIALS AND METHODS

Study of Symptoms

The infected cocoons and larvae were collected from Berhampore, West Bengal and carried to the laboratory in sterilized biodegradable polythene bags and the symptoms studied under hand lens and simple microscope.

Isolation and purification of pathogen from diseased parts

The collected larvae were washed with sterile distilled water and soaked in 70% alcohol to remove the surface impurities and cut into small pieces of 3-5 mm in size from the diseased portions, passed through 0.1% of $HgCl_2$ solution for one minute for surface sterilization and washed three times with three changes of sterile distilled water. The small pieces were blotted between sterile filter papers and aseptically plated on Potato Dextrose Agar (PDA). In each plate a single piece was placed and incubated at BOD (28± 1°C) for 7-days. After the appearance of mycelial growth it was transferred to fresh PDA slant. For purification of isolated pathogen, single hyphal tip method was followed.

Characterization and identification of the pathogen

Cultural characteristics on PDA and morphological character of the pathogen under compound microscope were recorded. The identification of the pathogens was done phenotypically following published key of Humber (2005).

Pathogenecity test of the pathogen

Pathogenecity test was done following the Koch postulate.

Isolation and characterization of antagonistic fungi

Isolation of antagonistic fungi from different rhizosphere soils from different regions of West Bengal, were done in the laboratory by serial dilution plating (Parkinson *et al.,* 1971). The fungal strains were identified following keys of Domsch *et al*. (1980) and Nagamani *et al.* (2006). The accession no. of isolates namely *T. viride* T 12, *T. viride* T 13 and *T. harzianum* are IARI herb. No. 108, 109, 114 respectively.

Test of mycoparasitism of isolated antagonistic fungi

Pathogens and antagonists were grown on separate sterilized petriplates containing PDA medium. After 5 days 5mm disc of young vigorously growing cultures of both pathogens and antagonists were uniformly cut off by cork borer and placed on the opposite point of a 9cm diameter sterilized petriplate containing 20 ml of PDA and incubated in a BOD at 28 ± 1°C for 7-days. A plate containing pathogen serves as control. After 7-days radial growth of the pathogen in both test plates were recorded. Antagonistic activity was measured by PIRG (Percentage of Inhibition of Radial

Growth). The PIRG was calculated following the formula of Topps and Wain (1957).

PIRG= (A-B)/A×100

A=Mean diameter growth in control

B=Mean diameter growth in given test.

Mechanism of action of biocontrol agents compound light microscope study

Observations on the mechanisms of hyphal interactions were carried out following method described by Chet *et al.* (1997). The sterilized glass slide coated with a thin layer of PDA is placed inside a pair of sterilized moist petriplate. Agar discs covered with mycelium of host fungi was placed on one end of PDA coated glass slide and discs with antagonist organism on the other end. All plates were incubated in a BOD at 28 ± 1°C for 7-days. In each case the antagonist and pathogen grew towards each other and hyphae intermingled on the slide. The slides were observed under high power (40 X) and oil immersion lens (100 X) of a compound light microscope (Olympus CX 31).

RESULTS AND DISCUSSIONS

Symptoms

White muscardine disease

While suffering from white muscardine, silkworm larvae become sluggish and inactive by losing appetite and also stop to move. The elasticity of its cuticle is lost and it becomes mummified by hardening. The entire body of the silkworm gets covered by white mycelium and powdery white conidia which ultimately leads to decay of the body (Fig 1). When a pupa is infected, it often mummifies, shrinks, wrinkles and gets engulfed in fungal mycelia coating. In an adult moth, the body hardens and the wings drop off. The symptoms recorded in these experiments are at par with the observations of previous workers (www. csrtimys.res.in).

Fig. 1. Silkworm infected with white muscardine disease

Cultural and microscopical characters of pathogen

Colonies are restricted in growth, white in colour, covering full plate in 10-12-days at 28^0C on PDA (Fig 2). It produces many dry, powdery conidia in distinctive white spore balls. Each spore ball is composed of a cluster of conidiogenous cells. Reverse plate is colorless, hyphae thin much branched. The conidiogenous cells are short and ovoid, terminate in a narrow apical extension called a rachis 4-5 µm. The rachis elongates after each conidium is produced, resulting in a long zig-zag extension. The conidia are single-celled, round to elliptic 3µm in diameter, haploid, and hydrophobic (Fig 3). After matching with the published key the fungus was identified as *Beauveria bassiana.*

Fig. 2. 7 days old culture plate of *Beauveria bassiana* isolated from white muscardine disease of silkworm

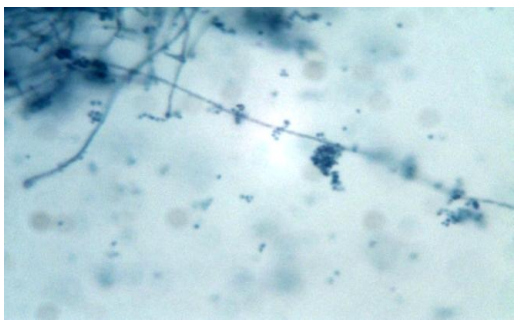

Fig. 3. Microscopic picture of Conidia and conidiophores of *Beauveria bassiana* (10X × 40X)

Green muscardine disease

The Green Muscardine Fungus (GMF) infects the larva, pupa and adult of the beetle. The white mass of the fungus can first be seen on the surface of the mummified body of the

beetle about 10 days from infection. The green colouration of the fungus appears after another 3 to 5 days.

Cultural and microscopical characters of pathogen

Colonies are fast growing reaching 9.00 cm diam. in 4-5 days at 28^0C on PDA (Fig 4). Hyphae floccose, white, during sporulation turned olive green to dark green. Reverse plate colorless. Sometime exude can found. Mycelium thin, branched, septate, 3µm wide. Chlamydospores ovoid with tapering end, intercalary or terminal, 15-18 µm in length and 10-12 µm in breadth. Conidiospores are produced in bunch cluster, abundant, each spore is round to ovoid 3-3.5 µm in diam (Fig 5). After matching with the published key the fungus is identified as *Metarhizium anisopliae*

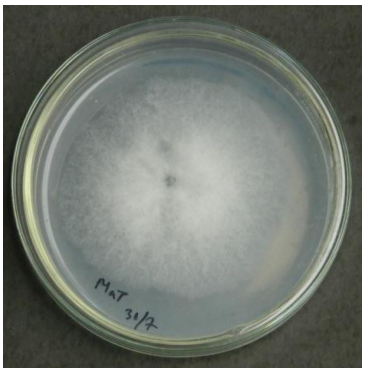

Fig. 4. 7-day-old culture plate of *Metarhizium anisoplae* isolated from green muscardine disease of silkworm.

Fig. 5. Microscopic picture of Conidia and conidiophores of *Metarhizium anisoplae* (10X × 40X).

Table 1. Percentage of Inhibition on Radial Growth (PIRG) of *Beauveria bassiana* and *Metarhizium anisoplae* by antagonistic fungi.

Antagonistic fungi	*Beauveria bassiana*		*Metarhizium anisopliae*	
	Average radial growth (cm)	Average PIRG	Average radial growth (cm)	Average PIRG
T. harzianum	2.50	71.88	2.86	68.02
T.viride (T12)	1.73	80.52	3.63	59.47
T.viride (T13)	3.30	62.89	3.16	64.68
Trichoderma spp.	2.83	68.16	3.76	57.98
Control	8.9	-	8.96	-
CD (p=0.05)		3.08		1.65
SE ±		1.26		0.67

Data presented in table 1 states that *Trichoderma viride* T 12 provides maximum *in vitro* control against *B. bassina* followed by *T. harzianum*, *Trichoderma* spp. and *T. viride* T13, whereas in case of *Metarhizium anisopliae*, *T. harzianum* provides maximum *in vitro* control, followed by *T. viride* T13, *T. viride* T12 and *Trichoderma* spp.

Within 4 days it was found that the colony of *Beauveria bassiana* was completely overgrown and engulfed by *T. viride* T12 (Fig6), *T. harzianum* and *Trichoderma* spp. The growth of *T. viride* T 13 slightly slower in comparison to *T. viride* (T12), *T. harzianum* and *Trichoderma* spp. It took 5 days to overgrow the colony of *Beauveria bassiana*. After monitoring the radial growth of both the pathogens it was found that *M. anisopliae* grows faster than *B. bassiana*.

Table 2. Abnormalities induced by antagonistic fungi on fungal pathogens.

Antagonistic fungi	*Beauveria bassiana*	*Metarhizium anisopliae*
T. harzianum	Adherence of spores of antagonistic fungi around the hyphae which induce granulation of fungal pathogen	Attachment and coiling of antagonistic hyphae on pathogenic fungi which induce granulation of fungal pathogen (Fig 8)
T. viride (T12)	Vacuolation, granulation and distortion of fungal pathogen were noted (Fig 10).	Coiling of antagonistic fungal hyphae around the fungal pathogen. Vacuolation, granulation and distortion of fungal pathogen were noted.
T. viride (T13)	Crowding and adherence of antagonistic fungi around the pathogenic hyphae.	Coiling of antagonistic fungal hyphae around the fungal pathogen (Fig 9) and growth of antagonstic fungi through the fungal pathogen.
Trichoderma spp.	Vacuolation, granulation and distortion of fungal pathogen were noted.	Vacuolation, granulation and distortion of fungal pathogen were noted.

Data presented in table 2 reveals that, microscopic observations after appropriate period of incubation showed coiling of *Trichoderma* hyphae over the hyphae of *B. bassiana* and *M. anisopliae* which is a clear evidence of hyperparasitism. Beside hyphal coiling, crowding and adherence of spores of *T. viride, T. harzianum* over the hyphae of *Beauveria bassiana* and *Metarhizium anisopliae* results deformations, granulation and vacuolation of pathogenic hyphae. Parasitism of fungi particularly mycoparasitism is a special mode of their existence for many species. Since the discovery that *Trichoderma* has great potential for biocontrol (Weindling, 1934), many researchers dealing with *Trichoderma* noticed that hyphae of the antagonists parasitized hyphae of other fungi *'in vitro'* and brought about several morphological changes like coiling, haustoria formation, disorganization of host cell contents and penetration of the host (Papavizas, 1985). Durrell (1986) has provided classical evidence of mycoparasitic activity of *Trichoderma* through phase contrast and electron microscopy. Similar evidences on the mechanism of hyperparasitic activity have been recorded by Chet *et al*. (1981) through advanced microscopic studies.

T. viride and *T. harzianum* (Pan and Ghosh, 1997; Ghosh, 2000, 2002; Ghosh and Chakraborty, 2012; Gveroska and Ziberoski, 2012; Prabhakaran, 2015; Tagram, 2015) species showed potential *in vitro* antagonistic activity against important plant pathogens viz. *Sclerotinia sclerotiorum, Pyricularia oryzae, Bipolaris oryzae, Alternaria solani. A. alternata*. *Phytophthora colocasiae, P. parasitica* var *piperina, Pythium aphanidermatum* and *Colletotrichum gloeosporoides*. Different species of *Trichoderma* were also applied for *in vitro* control of different fungal pathogens from mulberry (*Morus* sp.). For example, *Trichoderma harzianum* (Th-1) and *Trichoderma pseudokoningii* (Tp) have given significantly good result in *in vitro* control of the pathogen *Cercospora moricola* (leaf spot disease) (Sharma and Gupta, 2000), *Ceratelium fici* and *Pteridium mori* (black rust disease), *Fusarium solani* and *F. oxysporum* (root rot disease) (Kumari, 2014). But there is no available report on *in vitro* control of *Beauveria bassiana* and *Metarhizium anisopliae* through the application of *Trichoderma.* According to a recent survey, it is revealed that the loss is around Rs. 50 lakhs per annum in Chittor district of Andhra

Pradesh due to white muscardine disease (Kumareshan, 2003). Generally to combat the disease 30% chlorine, 2 % bleaching powder, bed disinfectant like ankush (Reddy and Rao, 2009), 0.028% deltamethrin solution, 5% malathion, 0.076% DDVP, dusting with 1-2% diethene M 45, Kaolin, Captan etc are used (www.karunadu.gov.in/sericulture). In 1999 and 2002, Nataraju and his coworkers of CSR&TI, Mysore tried to develop an integrated technology to control of silkworm diseases and used chlorine dioxide, Anukush and Vijetha as fungicidal components. Some pesticides are even carcinogenic and causing some human cancer such as colorectal cancer (Lee *et al.*, 2007), breast cancer (Abdalla *et al*., 2003), leukemia and non-Hodgkin's lymphoma in childhood (Meinert *et al.*, 2000). However, the potential impact on environment as well as health largely limits their application (Eckert *et al.*, 1994). Hence, to reduce the use or dose of chemicals, one possibility is to utilize the disease suppressing activity and plant growth promoting capacity of certain microorganisms in agrifields which should be highly ecofriendly. Such microorganisms are commonly referred to as biological control (biocontrol) agents (BCA). Beside that, not only a huge amount of money is lost behind the application of these chemical fungicides but also it kills a huge number of non target organisms and reduces biodiversity. On the other hand, i*n vitro* biological control of these renowned entomopathogenic fungi like *Beauveria bassiana* and *Metarhizium anisopliae* through *Trichoderma* is a completely new idea which can open a new vista of eco friendly approach to encounter white muscardine and green muscardine disease. Therefore it is a unique way to encounter the fungal pathogens of white and green muscardine disease of silkworm and to save the sericulture industry.

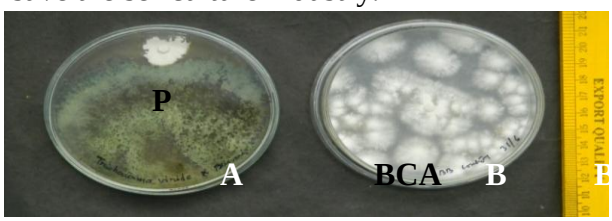

Fig. 6. Control plate of *B. bassiana* (B). *B. bassiana* treated with *T. viride* T 12 (A)-7 days old culture grown on PDA. P= pathogen (*B. bassiana*); BCA= bicontrol agent *(T. viride* T 12*)*

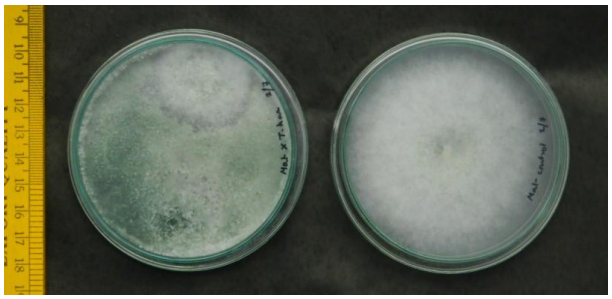

Fig. 7. Control plate of *M. anisopliae* (B). *M. anisopliae* treated with *T.harzianum* (A). (7 days old culture grown on PDA)
P= pathogen (*M. anisopliae*); BCA= bicontrol agent *(T. harzianum)*

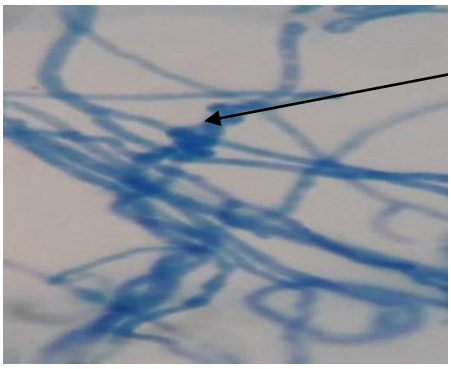

Fig. 8. Coiling of *T. harzianum* hyphae around the pathogen *M. anisoplae* (10X × 40X)

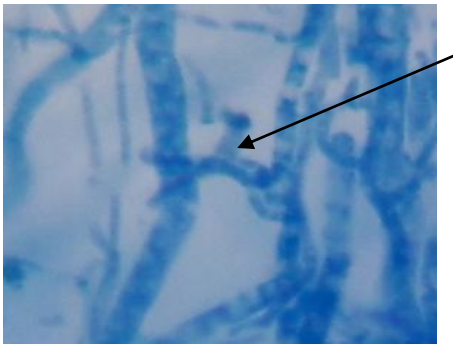

Fig. 9. Coiling of *T. viride* T 13 hyphae around *M. anisopliae* and hyphal bulging of the later. (10X × 100X)

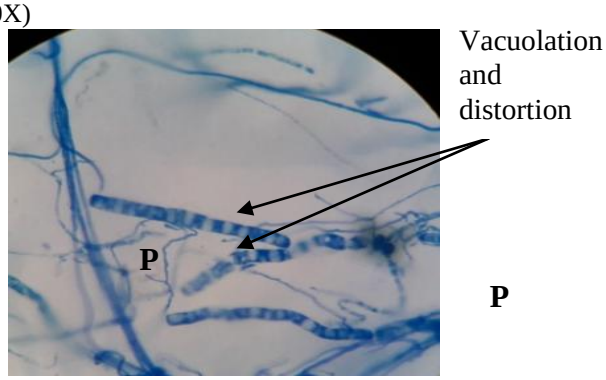

Fig. 10. Vacuolation and distortion of *B. bassiana* hyphae after coming in contact with *T. viride* T12 hyphae (10X × 40X)

Hyperparasitic nature of the biocontrol agents were confirmed by hyphal coiling of *T. harzianum* and *T. viride* over *M. anisopliae* as well as cellular changes like granulation, distortion and vacuolation of pathogenic fungi induced by different species of *Trichoderma*. It is evident that application of different species of *Trichoderma* provides significant *in vitro* control of *Beuveria bassiana* (C.O. of white muscardine disease) and *Metarrhizium anisoplae* (C.O. of green muscardine disease). Among our implemented biocontrol agents *T. viride* T 12 provides maximum *in vitro* control against *B. bassina* followed by *T. harzianum*, *Trichoderma* sp. and *T. viride* T13 (62.89%).Where as in case of *Metarhizium anisopliae, T. harzianum* provides maximum *in vitro* control, followed by *T. viride* T13, *T. viride* T12 and *Trichoderma* sp.. *In vitro* biological control of renowned entomopathogenic fungi like *Beuveria bassiana, Metarhizium anisoplae* through *Trichoderma* is a completely new idea which may be implemented *in vivo* and can unveil a new vista of eco friendly approach to encounter white muscardine and green muscardine disease by avoiding utilisation of environmental hazardous and toxic chemicals and thereby save the sericulture industry.

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