Title page An insight of anopheline larvicidal mechanism by a novel entomopathogen – *Trichoderma asperellum* (TaspSKGN2)

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Research Article

Keywords: Anopheles mosquitoes, Trichoderma asperellum, ME, hamocyte, entomopathogen , mechanisms, Pr1 protein, chitinase , protease, interaction

Posted Date: April 28th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-460909/v1

Version of Record: A version of this preprint was published at Scientific Reports on August 6th, 2021. See the published version at https://doi.org/10.1038/s41598-021-95310-1.

Abstract

Anopheline larvicidal property of *T. asperellum* has been found recently in medical science. The mechanisms of action exhibited by *T. asperellum* to infect mosquito larvae is the pivotal context of our present study. To infect an insect, entomopathogens must undergo some events of pathogenesis. We performed some experiments to find out the mechanisms of action of *T. asperellum* against anopheline larvae and compared its actions with other two well recognized entomopathogens like *Metarhizium anisopliae* and *Beauveria bassiana.* The methodology adopted for this includes Compound light and SE Microscopic study of host-pathogen interaction, detection of fungal spore adhesion on larval surface (Mucilage assay), detection of cuticle degrading enzymes (Spore bound pr1, Chitinase and Protease) by spectro-photometric method, Quantitative estimation of Chitinase and Protease enzymes, and determination of nuclear degeneration of hemocyte cells of ME treated larvae by *T. asperellum* under florescence microscope. Compound light microscopic studies showed spore attachment, appressorium and germ tube formation, invasion and proliferated hyphal growth of *T. asperellum* on epicuticle and inside of dead larvae. SEM study also supported them. After 3 hrs of interaction, spores were found to be attached on larval surface exhibiting pink coloured outer layer at the site of attachment indicating the presence of mucilage surrounding the attached spores. The enzymatic cleavage of the 4-nitroanilide substrate yields 4-nitroaniline which indicates the presence of spore-bound PR1 protein and it was highest (absorbance 1.298 ± 0.002) for *T. asperellum* in comparison with control and other two entomopathogens. *T. asperellum* exhibited highest enzymatic index values for both chitinase (5.20) and protease (2.77) among three entomomethogens. Quantitative experiment showed that chitinase enzyme concentration of *T. asperellum* (245 µg/mL) was better than other two *M. anisopliae* (134.59 µg/mL) and *B. bassiana* (128.65 µg/mL). Similarly Protease enzyme concentration of this fungus was best (298.652 µg/mL) among three entomopathogens. Here we have detected and estimated fragmentized nuclei of hemocyte cells by fluorescence microscopy in treated larvae with different ME doses of *T. asperellum*, and also observed that mosquito larvae exposed to 0.1mg/ml dose of ME showed maximum (100%) nuclear fragmentations of hemocytes and while 20, 45, 70 and 85% of nuclear deformities were recorded at 0.02, 0.04, 0.06 and 0.08 mg/ml concentration of ME. The knowledge of this work certainly will help in understanding of mechanism of action of *T. asperellum* for anopheline larval killing and consequently in eradication of malaria vector.

Introduction

Of late/recently the world is going through an extremely challenging and tough period as it is severely affected by several deadly mosquito borne diseases like Malaria, Dengue, Chikunguniya, etc. A thorough knowledge of the physico-chemical factors, which influence mosquito habitat on larval production, and a good understanding of the biological and ecological aspects of mosquito vector species are of great importance in the case of formulating effective plan and careful implementation of integrated vector control strategies by environmental management[1] . Recently Ghosh et al.[1] reported that there-adult mosquito aquatic niches parameters have a great role for the integrated mosquito control programme On

the other hand, chemical insecticides are randomly used to keep mosquitoes in control, but they have hazardous effects on environment and human health. So, bio-insecticides along with mosquito's larval aquatic niches can be a good alternative for chemical insecticides[2]. The survey, description and application of insect pathogens are global important[3]. Bio control agents like *B. bassiana* and *M. anisopliae* have been used for several years to fight insects by many workers [4-7], as they are natural enemies of agricultural pests and have a great role in maintaining ecological balance[8, 9]. But entomopathogenecity of *Trichoderma longibrachiatum* and *Trichoderma asperellum* has been established in our laboratory as first reports in previous studies[10,11] . *T. asperellum* is a well known fungus in agriculture. It is regularly used in agriculture for many years both as bio control agents to curb plant pathogenic microbes and plant growth promoting agents, but anopheline larvicidal efficacy of *T. asperellum* (T.aspSKGN2) (GenBank Accession No. MG719999.1) is novel. The mechanisms of action exhibited by *T. asperellum* to infect mosquito larvae is the pivotal context of our present study. To infect an insect, entomopathogens must undergo some infection processes. To begin infection processes, at first fungal spores have to attach to the host surface by secreting adhesives[12]. Detection of fungal mucilage for attachment of spore to host surface is an important criterion to experiment fungal mechanism of infection. After successful attachment of spore, fungi have to invade insect cuticle either by mechanical process or enzymatic degradation, through formation of appresorium and then infection peg [13]. The main components of insect cuticle are chitin and other proteins [14]. Virulence of fungal pathogens can be determined by assaying enzymatic activities related to infection pathways, such as spore bound protease (Pr1), chitinases[15,16], etc. After penetrating the host body through invasion of cuticle, the entomopathogens secrete some toxic compounds inside haemocoel or other tissues of the larval body[17]. Toxic compounds may reduce the insect Phenol Oxidase (PO) content and degenerate insect hemocytes, causing loss of insect immunity[18]. Study of cytotoxicity to insect immune cells is a parameter of assaying insecticidal efficacy. Although the mechanisms of entomopathogenecity of other entomopathogenic fungi are well known to us, the mechanism of novel entomopathogen *T. asperellum*, as reported by us previously[11], has hardly been explored. There is a significant research lacuna in the way entomopathogen *T. asperellum* functions; its process of attachment into the outer cell of the cuticle of mosquito larvae and other pathogenicity processes. This research work takes up this research gap as its primary objective and explores further to gather knowledge about methods and mechanisms of novel entomopathogen *T. asperellum*. The consolidated objectives of this research work are as follows: i) observation of host-pathogen interaction by compound light and Scanning Electron Microscopy, ii) detection of mucilage on *T. asperellum* spore surface at attachment site on larval surface, iii) detection of spore bound pr1 (Pathogenesis related protein), chitinase and protease (caseinase) enzymatic activities of *T. asperellum,* iv) comparison of enzymatic activity of *T. asperellum* with known entomopathogens *i.e. Beauveria bassiana* (GenBank Accession No. KM604668.1) and *Metarhizium anisopliae*. and v) observation of nuclear morphology of hemocytes cells of ME treated larvae and percentages of hemocyte degradation Our study may provide the effective insights on mode of entomopathogenecity exhibited by *T. asperellum.*

Results

Compound microscopic determination of lethality

Fungal spore treated mosquito larvae were taken out at different times and stained with lactophenol cotton blue to observe host-pathogen interaction. After 5 hrs of interaction, spores were found to be germinated on larvae. Appresorium and infection peg were observed after 8 hrs of interaction. Proliferated hyphal growth on epicuticle of dead larvae was observed after 15 hrs of infection (Fig. 1).

Scanning Electron Microscopic Study

SEM study revealed the hyphal proliferation on host epicuticle layer (Fig. 2).

Mucilage assay

Fungal spore treated mosquito larvae were taken out at different times and stained with Ruthenium red (0.1%) to detect the spore adhesion and the presence of mucilage during the attachment process. After 3 hrs of interaction, spores were found to be attached on larval surface exhibiting pink coloured outer layer at the site of attachment (Fig. 3) indicating the presence of mucilage surrounding the attached spores.

Enzymatic assay for spore-bound Pr1

Freshly harvested conidia of fungal isolates inoculated with 0.1M Tris-Cl supplemented with 1mM succinyl-ala-ala-pro-phe-p-nitroanilide, exhibited yellow aqueous phase after reaction. The enzymatic cleavage of the 4-nitroanilide substrate yields 4-nitroaniline (yellow colour under alkaline condition) which indicates the presence of spore-bound PR1 protein (Fig. 4). Experiment was done in triplicates. Absorbance was taken at 405 nm and presented in the table 1. Highest absorbance (1.298±0.002) was recorded for *T. asperellum* in comparison with control and other two entomopathogens. It signifies that highest PR1 protein exits in spore of this fungus.

Table 1. Detection of spore bound Pr1 protein for different fungal isolates by spectro-photometric method.

Fungal Isolate	Absorbance (OD value in nm with SD)(Mean±SD)
T. asperellum	1.298±0.002
M. anisopliae	1.171±0.009
B. bassiana	0.565±0.001
Control	0.103±0.001

Chitinase detection

Detection of chitinase by plate assay method

Preselected fungal isolates showed distinct enzyme hydrolytic zone in chitin amended Czapek Dox agar plates after five days of inoculation (Fig. 5). Relative enzymatic index was calculated for each fungus and presented in the table 2. Hydrolytic zone diameter (8.4 cm) was noted best by *T. asperellum* among three entomopathogens *T. asperellum* exhibited highest enzymatic index value (5.20) among three (Table 3 and Fig. 6).

Table 2. Relative Enzymatic Index of *T. asperellum*, *M. anisopliae* and *B. bassiana* in Chitin amended agar plates.

Fungal isolates	Hydrolytic zone diameter (cm) (Mean ±SD)	Colony diameter (cm))(Mean ±SD)	REI (Relative Enzymatic Index)
T. asperellum	8.4± 0.68	2.0±0.17	5.20
M. anisopliae	3.2± 0.38	0.78± 0.03	5.10
B. bassiana	2.5± 0.11	0.7±0.002	4.57

Table 3. Detection of Chitinase for different fungal isolates by spectrophotometric method.

Fungal isolates	Absorbance (OD value in nm with mean ± SD)
T. asperellum	0.899±0.008
M. anisopliae	0.282±0.007
B. bassiana	0.202±0.007
Control	0.064±0.002

Enzymatic assay for chitinase

Fungal culture filtrate of fully grown preselected fungi (*T. asperellum*, *B. bassiana* and *M. anisopliae*) collected from Chitin amended Czapek Dox broth were subjected to enzyme substrate reaction by adding PNG as substrate (Fig. 7). Experiments were done in triplicates. Absorbance was taken for each sample and OD values were presented in the table 3. Highest absorbance (0.899±0.008) was recorded for *T. asperellum* in comparison with others.

Protease Detection

Detection of protease by plate assay method

Preselected fungal isolates (*T. asperellum, M. anisopliae*, *B. bassiana*) exhibited distinct enzyme hydrolytic zone in casein amended Czapek Dox agar plates after five days of inoculation (Fig .8). Relative

enzymatic index was calculated for each fungus and presented in the table 4. *T. asperellum* exhibited highest enzymatic index value which is 2.77 amongst three entemomethogens.

Table 4. Relative Enzymatic Index of *T. asperellum*, *M. anisopliae* and *B. bassiana* in Casein amended Czapek Dox agar plates.

Fungal isolates	Hydrolytic zone diameter (cm) (Mean ±SD)	Colony diameter (cm) (Mean ±SD)	REI (Relative Enzymatic Index)
T. asperellum	8.5± 1.78	4.8±1.56	2.77
M. anisopliae	1.4±0.11	0.8±0.013	2.75
B. bassiana	0.6±0.02	0.4±0.01	2.5

Enzymatic assay for Protease

Fungal culture filtrate of fully grown preselected fungi (*T. asperellum*, *B. bassiana* and *M. anisopliae*) collected from Casein amended Czapek Dox broth were subjected to enzyme substrate reaction by adding casein powder as substrate. REI of proteases was represented in Fig. 9. REI of *T. asperellum* was best among three entomopathogens. Absorbance was taken for each sample and OD values were presented in the table 5. The absorbance of *T. asperellum* was best (0.092±0.004) among three entemopathogens,

Table 5. Detection of protease for different fungal isolates by spectrophotometric method.

Fungal isolates	Absorbance (OD value in nm) (Mean±SD)
T. asperellum	0.092±0.004
M. anisopliae	0.089±0.003
B. bassiana	0.090±0.001
Control	0.061±0.001

Quantitative estimation of Chitinase and Protease enzymes

Chitinase estimation

The estimated concentrations of chitinases from culture filtrates of different isolates were calculated using standard curve, constructed with absorbances of different known concentrations of BSA (Fig. 10). The chitinase concentrations of three isolates were presented in the table 6. The data (Table 6) showed that chitinase enzyme concentration of *T. asperellum* (245 µg/mL) was better than other two *M .anisopliae* (134.59 µg/mL) and *B bassiana* (128.65 µg/mL).

Table 6. Absorbances and concentrations of chitinase enzymes in culture filtrates of three isolates.

Fungal isolates	Absorbance (OD value in nm)(Mean±SD)	Concentration(µg/mL) of enzyme (Mean±SD)
T. asperellum	0.273±0.006	245.204±30.4
M. anisopliae	0.116±0.003	134.598±26.6
B. bassiana	0.124±0.016	128.659±22.78

Protease estimation

The estimated concentrations of proteases from culture filtrates of different isolates were calculated using standard curve, constructed with absorbances of different known concentrations of BSA (Fig. 10). The protease concentrations of three isolates were presented in the table 7. The data (Table 8) showed that protease enzyme concentration of *T. asperellum* (298.652 µg/mL) was best in comparison with other two *M. anisopliae* (263.02µg/mL) and *B. bassiana* (230.358 µg/mL) and control.

Table 7. Absorbances and concentrations of protease enzymes of three isolates in culture filtrates.

Fungal isolates	Absorbance (OD value in nm) (Mean ± SD)	Concentration(µg/mL) of enzyme (Mean ± SD)
T. asperellum	0.345±0.033	298.652± 54.09
M. anisopliae	0.297±0.055	263.02± 51.56
B. bassiana	0.253±0.054	230.358± 42.43

Table 8. Percentage of deformed nuclear hemocytes at different ME doses of *T. asperellum*.

Concentration of ME (mg/ml)	Percentage (%) of hemocytes with deformed nuclei
0.02	20e
0.04	45d
0.06	70c
0.08	85b
0.1	100a
Control	10f

Note: Different letters in different rows indicate that they are statistically Different as per Duncan analysis ($p<0.05$)

Determination of nuclear deformities of treated larval hemocytes

DAPI stained, treated (ME of different doses) mosquito larval hemocytes exhibited increased nuclear fragmentations in dose-dependent way (Fig. 11 b-f). Degenerative nuclei were observed from different doses of ME (Fig. 11b-f) treated larval hemocytes, compared with control which evinced normal spherical shaped nucleus (Fig. 11a). Percentage of number of hemocytes with nuclear deformities grew with increasing doses in comparison with control (Table 8). Mosquito larvae exposed to 0.1mg/ml dose of ME showed maximum nuclear fragmentations and 100% of hemocytes exhibited nuclear deformities in this dose. 20, 45, 70 and 85% of nuclear deformities were recorded at 0.02, 0.04, 0.06 and 0.08 mg/ml concentration of ME.

Schematic outline of host pathogen interaction

The mechanism of entomopathogenic actions of *T. asperellum* on anopheline larvae was schematically outlined in Fig (12).

Discussion

In this present study, insect killing mechanism of a novel entomopathogen *Trichoderma asperellum* has been established and concentrations of secreted cuticle degrading enzymes of *T. asperellum* were compared with two known entomopathogens which were *B. bassiana* and *M. anisopliae* [19, 20]. To establish a successful attachment to the host surface, fungi have to secrete adhesives containing carbohydrates with protein moiety[12] . Previous study supports that spore tip mucilage is responsible for attachment of fungal spore to the host surface [21] . Mechanism of spore adhesion on larval surface is determined by detecting the presence of mucilage at spore attachment site on host surface, using mucilage specific dye, Ruthenium Red [22]. Our study also validates that this fungus adapts to secret mucilage on spore wall for host attachment. Hyphal growth of the fungus on cuticle of mosquito larvae is observed by compound microscopy and SEM study. To invade the insect cuticle, which is mainly composed of Chitin and several other proteins, entomopathogens must secrete chitinase and protease enzymes [23]]. Mechanism of enzymatic degradation of larval cuticle for hyphal penetrance by this fungus inside larval body is confirmed by the detection of spore bound pr1 (Pathogenesis related protein), chitinase and protease enzymes which are secreted by the fungus. In enzymatic assay, *T. asperellum* exhibits highest absorbance in end product of enzyme-substrate reaction confirming the presence of spore bound pr1in appropriate amount, chitinase and protease with highest quantity amongst the other two. In agar plate assay for Protease and Chitinase, highest relative enzymatic index is also recorded for *T. asperellum*. Highest concentrations of chitinases and protease enzymes are exhibited by *T. asperellum* amongst other two in quantitative protein estimation. The larvae generally respond to the fungal infection by humoral mechanisms. After hyphal penetrance through larval cuticle, fungi secrete toxins, inside the larval body, which deteriorate larval immunity to complete the infection process[24]. Phenol oxidase (PO) of insect hemolymph and cuticle generally acts as a part of innate immunity of insect against infecting microbes. Larval PO is associated with melanin biosynthesis and haemocyte

production for self defense; decreased PO indicates reduction of immunity which provide favourable conditions for pathogens growth inside the insect's body[25]. In our previous study, we have reported decreased larval phenol oxidase content in ME treated larval hemolymph and cuticle, in comparison with control[11] . For mycochemistry of ME , the crude ME was fractionized, and each fraction was evaluated against anopheline larvae. MF8, out of 12, was most lethal to anopheline larvae. GC-MS analysis of MF8 confirmed us that 49 compounds were present Out of these compounds, seven compounds were recorded as insecticidal or mosquitocidal in work of previous workers[11]. In our earlier work[11], these seven compounds were noticed to be present in high abundance in MF8, Hemocyte, circulating immune cell, which is a vital component of larval innate immunity, destroys fungal pathogens by phagocytosis[26]. In *Drosophila* larvae, hemocytes serve immunological protection by melanizing and engulfing microbess and producing antimicrobial peptides. In addition, these immune cells by phagocytosis scavenge the apoptotic cells and play an important role during metamorphosis of this fly [27-29] . Here we have detected fragmentized nuclei of hemocyte cells by fluoroscence microscopy in treated larvae with different ME doses of *T. asperellum,* and also observed increased percentage of hemocyte degeneration in dose-dependent manner. Our observation reveals that the mode of action of the fungus weakens larval immunity resulting in the larval death. Furthermore, comparative analysis of enzymatic assays of *T. asperellum* with *B. bassiana* and *M. anisopliae* exhibits that *T. asperellum* secretes highest concentrations of cuticle degrading enzymes amongst the other two. In our previous study[11] we have shown that *T. asperellum* have lower LD_{50} and LT_{50} value amongst other known entomopathogens. Mechanistic study and comparison of enzymatic assay with other two also validate that *T asperellum* can be more effective than other two well known entomopathogen in controlling mosquito larvae.

After meticulous examination in the laboratory this research work comes to a fruitful conclusion which would contribute to future research works in this specific field. The mechanistic study of *T. asperellum* exhibits following mode of infections to kill anopheline larvae: i) Secretion of mucilage from spore for its attachment on larval surface ii) After germination of spore, penetration of insect cuticle by secretion of cuticle degrading enzymes (spore bound Pr1, Chitinase and Proteases) through infection peg iii) After penetration, secretion of toxins inside larval body which decrease larval phenol oxidase and degenerate hemocyte cells by nuclear fragmentations causing larval immunity breakdown followed by death. Application of the fungus as a new effective bio-control agent to eradicate anopheline larvae can open up new direction of *Trichoderma* research as entomopathogen and play a major role in mosquito vector control and disease management programme.

Materials And Methods

Compound microscopic study of host-pathogen interaction

Mosquito larvae treated with LD_{50} dose of *T. asperellum*[11] were taken out from the treatment set with needle and stained with lactophenol cotton blue solution in a grease free slide and mounted with cover-

slip. Slide was observed under compound microscope to detect mosquito-fungi (Host- pathogen) interaction.

Scanning electron microscopic study of host-pathogen interaction (SEM study)

T. asperellum spore treated infected mosquito larvae were subjected to Scanning Electron Microscopy (SEM) as demonstrated by Campos et al[30] .

Detection of Fungal spore adhesion on larval surface (Mucilage assay)

Twenty anopheline larvae were exposed to LD_{50} dose (2.68×10^7 conidia/mL) of *T. asperellum* spore as estimated in our early paper[11] . Larvae were taken from treatment set at each 1hr of interval in grease free slides to examine the fungal spore adhesion on larval surface. 100 µL of 0.1% Ruthidium Red (Mucilage specific stain [22] was applied to each slide to detect the presence of mucilage on spore surface at spore attachment site on larval cuticle under compound microscope. Presence of mucilage can be detected by alteration of red colour of the dye into pink[31] .

Detection of Cuticle degrading enzymes (Spore bound pr1, Chitinase and Protease):

Spore-bound Pr1 enzyme assay

Spore bound Pr1 activities of preselected fungi (*T. asperellum*, *B. bassiana* & M. *anisopliae*) were assayed following the modified protocol of Shah et al.[32] . Fungal isolates (isolated fungal) were grown in PDA plates and ten milligrams of conidia were harvested after definite incubation period. Conidia of each fungus were inoculated in 1 mL of 0.1M Tris-Cl supplemented with 1mM succinyl-ala-ala-pro-phe-p-nitroanilide ($C_{30}H_{36}N_6O_9$)(Sigma-Aldrich) and incubated for 5 min at room temperature (28±1ºC).After incubation, centrifugation of the sample was done at 12,000 g for 10 min at 4 ºC. The yellow aqueous phase was collected after separating conidia from the sample and transferred to wells in a flat-bottom microtiter plate. Absorbance was taken at 405 nm using ELISA (Microtiter) reader (Biorad, USA). Experiments were done in triplicates for each fungus. Buffer substrate was used as control for each set.

Chitinase enzyme assay

Detection of chitinase by plate assay method

Preparation of colloidal chitin

To prepare colloidal chitin from chitin flakes, modified protocol of Hsu and Lockwood [33] was followed. In a 100 ml beaker, 20 ml HCl was taken and 1g of chitin flakes was added slowly into it in the ratio of 20:1 and placed in a state of continuous stirring overnight at 4 ºC upon a magnetic stirrer. After overnight stirring, the entire solution was added to 400 mL of cold distilled water (20 volumes) under continuous stirring. Chitin flakes was converted into colloidal form in this process. Then the solution was centrifuged at 2000 rpm for 15 min at 4℃ and supernatant was discarded. Thus, the precipitate obtained, was

colloidal chitin which was highly acidic. The colloidal chitin was washed with cold distilled water repeatedly until the final pH become 7.0.

Preparation of growth medium

In a 1000ml beaker, 500ml of Czapek Dox agar medium ($NaNo_3$: 1g; KH_2PO_4 : 0.5g; $MgSO_4$, $7H_2O$: 0.25g; KCl : 0.25g; $FeSO_4$, $7H_2O$: 0.05g; Agar: 10g) was prepared. 1% of colloidal chitin (5ml) was suspended to it. The medium was sterilized by autoclave and poured in petridishes under sterile condition in Laminar Airflow Chamber.

Inoculation of fungal strains

Previously isolated fungal strains of *Trichoderma asperellum, Beauveria bassiana* and *Metarhizium* sp. were inoculated in the Czapek Dox agar medium containing colloidal chitin under sterile condition by hyphal tip inoculation method and incubated in B.O.D at 28±2 ºC.

Enzyme hydrolytic zone detection

After 3 days of inoculation, each petridish was flooded with Grams Iodine solution to detect the enzyme hydrolytic zone [34]. Grams iodine binds with soluble, unhydrolyzed chitin forming brown colour complex. Chitinase activity was visualized by detecting clear hydrolytic zone surrounding the fungal colony. Clear zone was formed due to hydrolyzation of soluble chitin by the activity of chitinase enzyme where grams iodine doesn't bind. Relative Enzymatic Index was determined with following formulae[35] by calculating hydrolytic zone diameter and fungal colony diameter.

Relative Enzymatic Index (REI):

$$\frac{Zone\ diameter + Colony\ diameter}{Colony\ diameter}$$

Detection of Chitinase in broth culture:

Preparation of Czapek Dox broth with colloidal chitin

Czapek Dox broth was prepared as described above. 1% of colloidal chitin was added to it following modified protocol of Murthy and Bleakley[36].

Inoculation of fungal strain

Previously isolated fungal strains of *Beauveria bassiana*, *Metarhizium anisopliae*, *Trichoderma asperellum* were inoculated in the Czapek Dox broth medium amended with colloidal chitin under sterile condition and incubated in Shaker B.O.D at 150 rpm at 30±2ºC for five days.

Enzymatic assay for chitinase

To assay chitinase enzyme activity standard protocol was followed[37,.38]. Chitinase produces p-nitrophenol (yellow coloured compound) in reaction with PNG. In a 96 well microtitre plate, 10 µL of fungal culture filtrate was taken from each set. 10 µL of 10 mM PNG (p-nitrophenyl β-D-N glucosaminide) and 30 µL of 0.1M PBS (pH: 6) were added into it. Here PNG was used as substrate. Six replicas were taken for each fungus. The micro titer plate was incubated at 37 ºC for 1hr. After that, reactions were stopped by adding 50 µL of Na_2CO_3 in each well. Absorbance was recorded at 415 nm.

Protease enzyme assay

Detection of protease by plate assay method

Preparation of Czapek Dox agar medium with casein powder

Czapek Dox agar medium was prepared and sterilized. 1% (w/v) of Casein powder was mixed in the medium after the medium became little cold, and poured into the petridishes. Modified protocol of Parida et al.[35] was followed.

Inoculation of fungal strain

Previously isolated and purified fungal strains of *Beauveria bassiana*, *Metarhizium* sp., *Trichoderma asperellum* and *Penicillium verrucosum* were inoculated in the Czapek Dox agar medium containing casein powder under sterile condition by hyphal tip inoculation method and incubated in B.O.D at 27±2ºC.

Enzyme hydrolytic zone detection

Enzyme hydrolytic zone was detected from culture plate of each fungus by pouring 5ml of 10 % TCA after five days of fungal inoculation following the modified method of Medina and Baresi [39],[29] and Al Nahdi[40] .

Detection of protease in broth culture

Preparation of Czapek Dox broth medium with casein powder

Czapek Dox broth medium was prepared and sterilized. Casein powder (1%) (w/v) was added into the medium after little cooling.

Inoculation of fungal strains

Previously isolated and purified fungal strains of *Beauveria bassiana*, *Metarhizium anisopliae*, *and Trichoderma asperellum* were inoculated in the Czapek Dox broth medium containing casein powder under sterile condition by hyphal tip inoculation method and incubated in B.O.D at 30±2ºC.

Enzymatic assay for protease

To determine protease activity, modified procedure of Tsuchida et al.[41] was followed. After five days of inoculation 200 µL of culture filtrate was taken out from each fungal culture in separate eppendroff tube (2 mL). 500 µL of 1 %(W/V) casein powder suspended in 50mM PBS were added as substrate for each set. Then each set was incubated for 15 mins in water bath at 45ºC for enzyme substrate reaction. Then 1 mL of 10 %TCA was added in each set to terminate the reaction. After that, each reaction mixture was centrifuged at 10000 rpm for 15 minutes. 500 µL of supernatant was taken from each set in separate eppendroff tube. Then the supernatant was mixed with 1mL of 0.4 M Na_2Co_3 and 0.5 mL of 3 fold diluted Folin-ciocalteu reagent and incubated at room temperature (37 ºC) in the dark for 30 minutes. 100 µL of each resulting solution was taken in a 96 well microtitre plate, and absorbance of developing blue color was measured at 660 nm against reagent blank using distilled water in place of culture filtrate.

Quantitative estimation of Chitinase and Protease enzymes

Chitinase quantification: Fungi were inoculated in 50 mL of 1% Chitin amended media (as described before) and incubated at shaker B.O.D at 150 rpm and 28±2 ºC temperature. After five days of inoculation, 5 ml of culture filtrates were taken from the media and the total proteins were recovered by Acetone precipitation [42] (sample: Acetone= 1:2) in 15 ml falcons. The precipitated proteins were subjected to quantitative estimation. A standard curve was prepared with different known concentrations of BSA by spectrophotometric method[43]. Concentration of Chitinase enzymes were calculated using different known concentrations of BSA as standard by MS EXCEL, 2007.

Protease quantification: Fungi were inoculated in50 mL of 1% casein amended media (as described before) and incubated at shaker B.O.D at 150 rpm and 28±2 ºC temperature until the colour of casein disappeared. After that 5ml of culture filtrates were taken from the media and the total proteins were precipitated by Acetone cut [42] (sample: Acetone = 1:2) in 15 ml falcons. The precipitated proteins were quantified as described above.

Determination of nuclear degeneration of hemocyte cells of ME treated larvae by *T. asperellum*

Mosquito larvae treatment with ME

Anopheline larvae were treated with different doses (0.04 mg/ml, 0.06 mg/ml, 0.08 mg/ml and 0.1 mg/ml) of methanolic extract (ME) with control as reported in an earlier study[11] . After 8 hrs of interaction larvae (30) were extracted, washed by distilled water and sterilized with sodium hypochlorite (5%).

Collection of larval haemocytes cell

For the collection of haemocytes the cuticle of each larvae were disrupted using two micro syringe at the intersection of head and thorax and the hemolymph were released by applying gentle pressure on the thorax with the help of microsyringe. The whole procedure was done in a grease free slide into drop of 40

µL PBS with 0.07% phenylthiourea (PTU). After collecting the hemolymph of 10 larvae in PBS, it was transferred into a micro-centrifuge tube with the microinjection syringe. The procedure was repeated to collect the hemolymph of total 30 larvae from each set. The tubes from each set were then centrifuged at 300 g for 15 min at 4 ºC. The supernatant was discarded and the cell pellet was re-suspended in 20 µL of PBS containing 0.07% PTU[44] .

Staining of nucleus of larval haemocytes and fluorescence microscopy

The Cell suspension was placed in a grease free slide and10µl of aqueous DAPI solution (1 µg/ml) were added [45,46]. The solutions were mixed properly by pipetting. Solution mixture were covered with a cover slip and incubated in dark for 15 min. After that, the slides were placed under inverted fluorescence microscope (Olympus-CKX53) for observation using Q imaging software.

Declarations

Acknowledgement Authors acknowledge DSTBT (WB) for financial support (Grant No 504/ST/P/S&T/9G/3/13/2015) to run the experiments and also Principal, RKMVC College, Rahara for lab. facilities.

Author Contributions S.K.G. has planned research design and written manuscript. D.P. has done some experimental works , literature collection and prepared Fig 1-6, A.M has done some experimental work data collection and data analysis and prepared Fig7-12. All authors checked and reviewed the Ms.

Funding source Department of Science & Technology and Biotechnology, Government of West Bengal has financed (Grant No 504/ST/P/S&T/9G/3/13/2015) to run the research work. Financer has no role in research design and publication of work.

Competing interests

Authors have no financial and non-financial competing of interests

Data Availability Statement

Data are available from www.nature.com.

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Figures

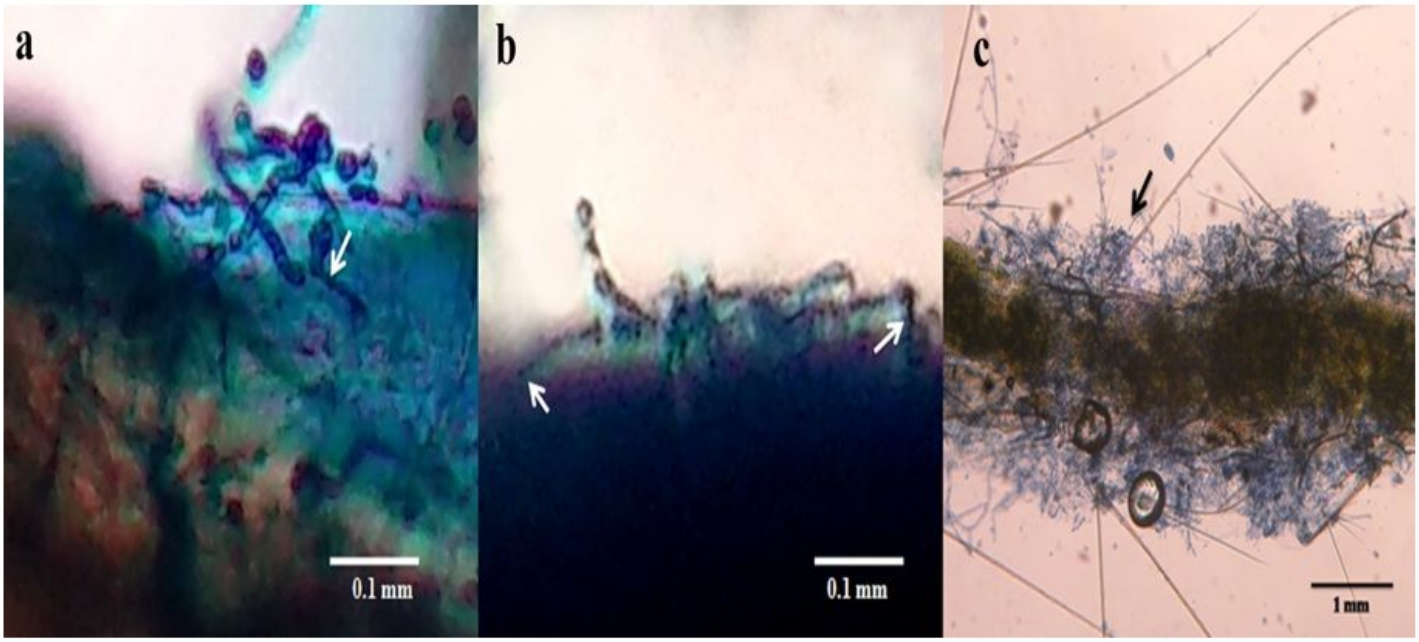

Figure 1

Interaction of T. asperellum spore with anopheline larvae under compound microscope (Olympus CX-31). a. Germination of fungal spore and germ tube formation on larval surface (40X). b. Cuticle penetration by formation of appresorium and infection peg (40X). c. Hyphal proliferation of fungus on dead larvae (10X).

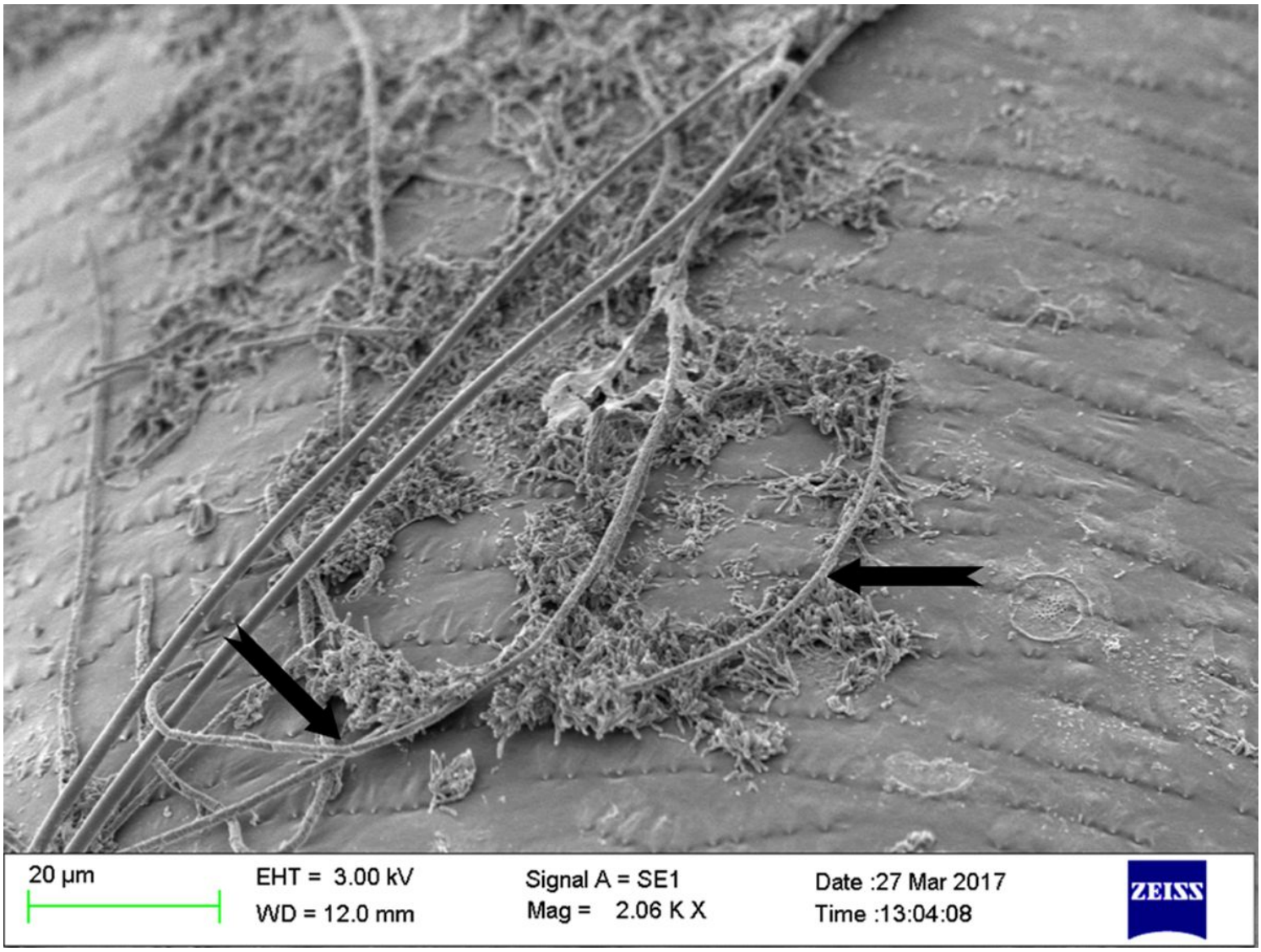

Figure 2

Scanning Electron Micrograph of proliferation of fungal hyphae on epicuticle of larvae.

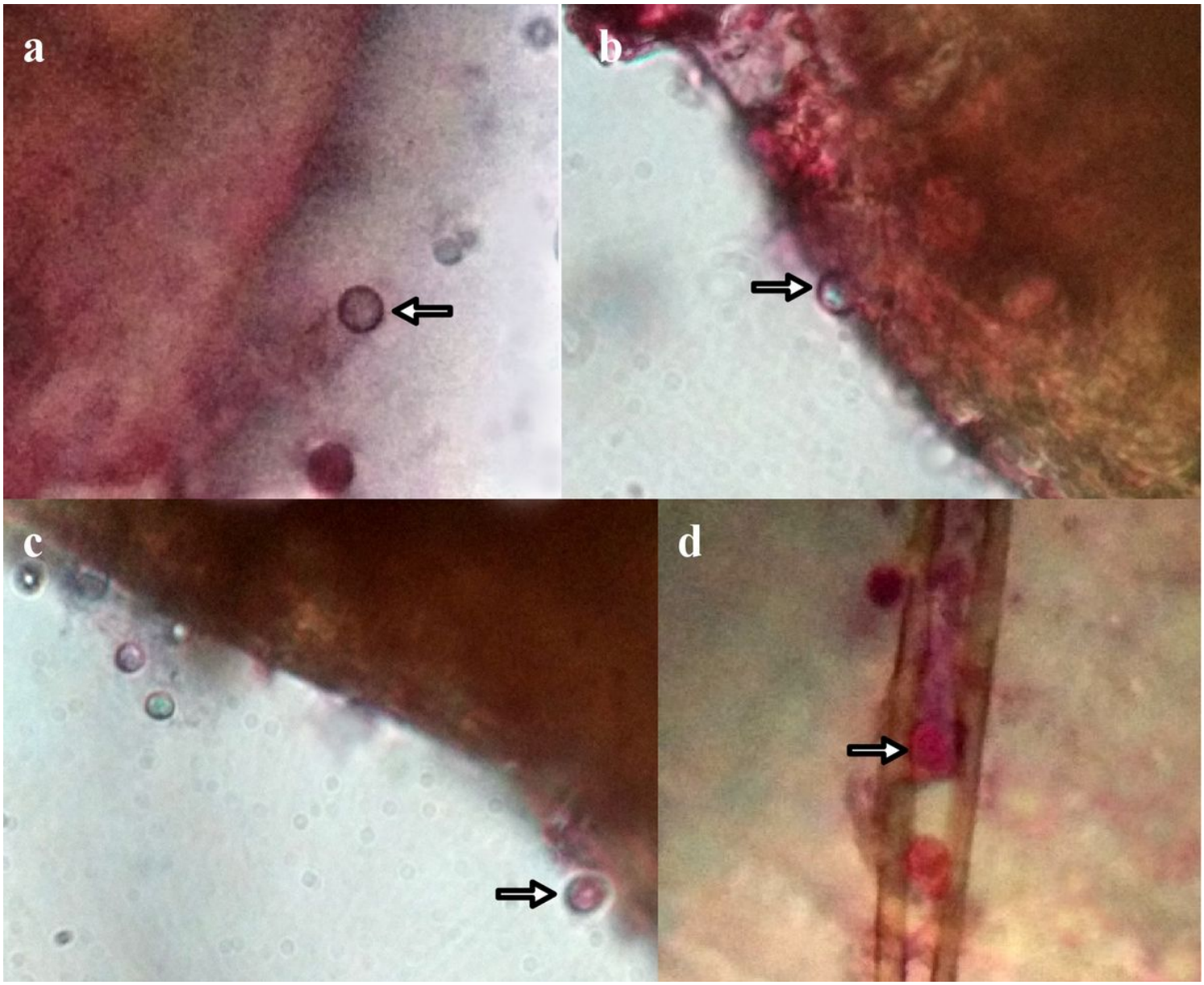

Figure 3

Adhesion of fungal spore on host surface under compound microscope (Olympus CX-31). a. b. c. Fungal spore attachment on different surfaces of epicuticle of larvae, d. attachment of spores on larval appendages, detected by mucilage specific dye (Ruthenium red).

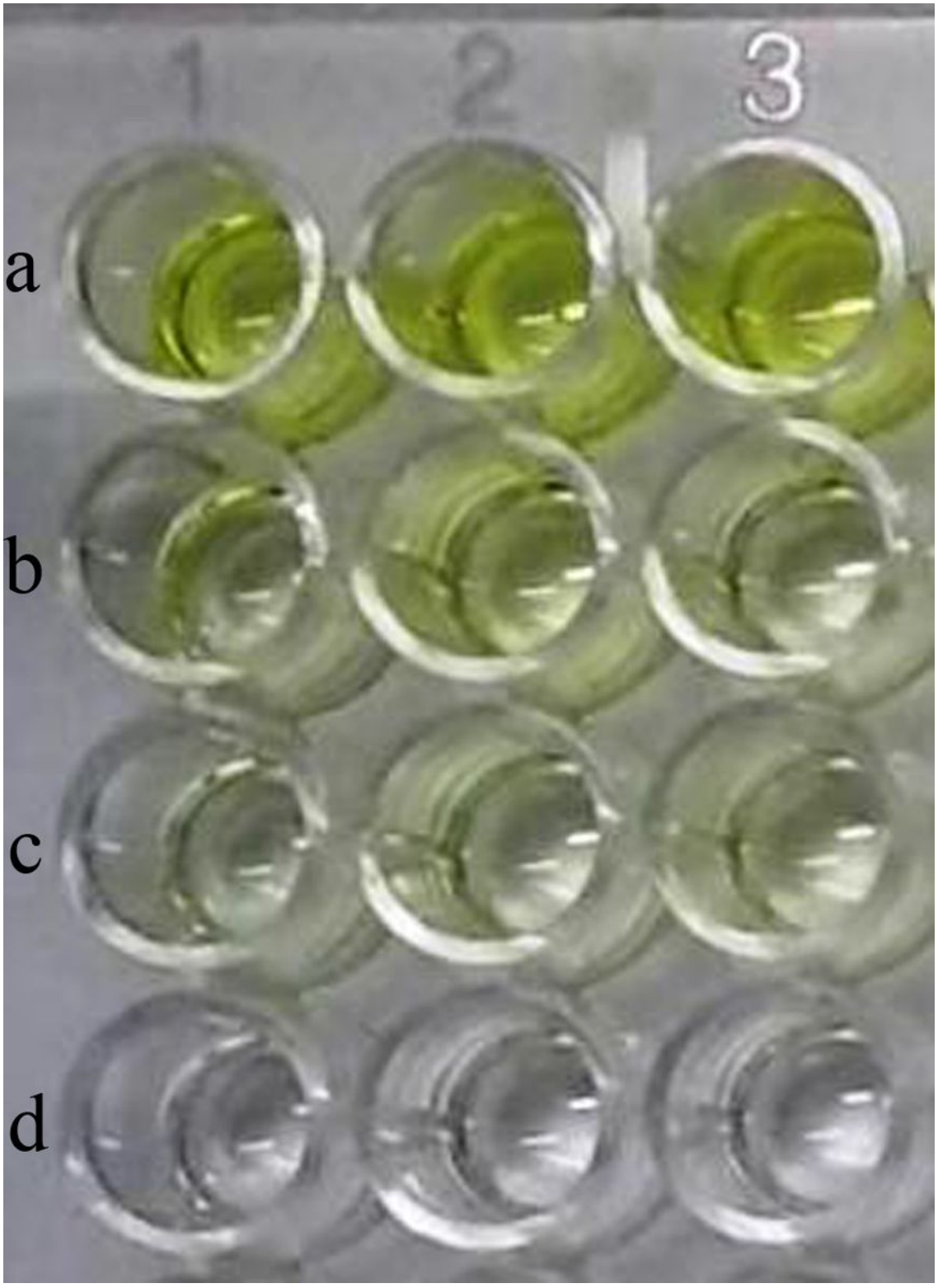

Figure 4

Formation of yellow coloured product (4-nitroaniline) after reaction of 4-nitroanilide with Pr1 protein, extracted from spore surfaces of three fungal isolates. a.T. asperellum, b.B. bassiana,c.M. anisopliae, d. control

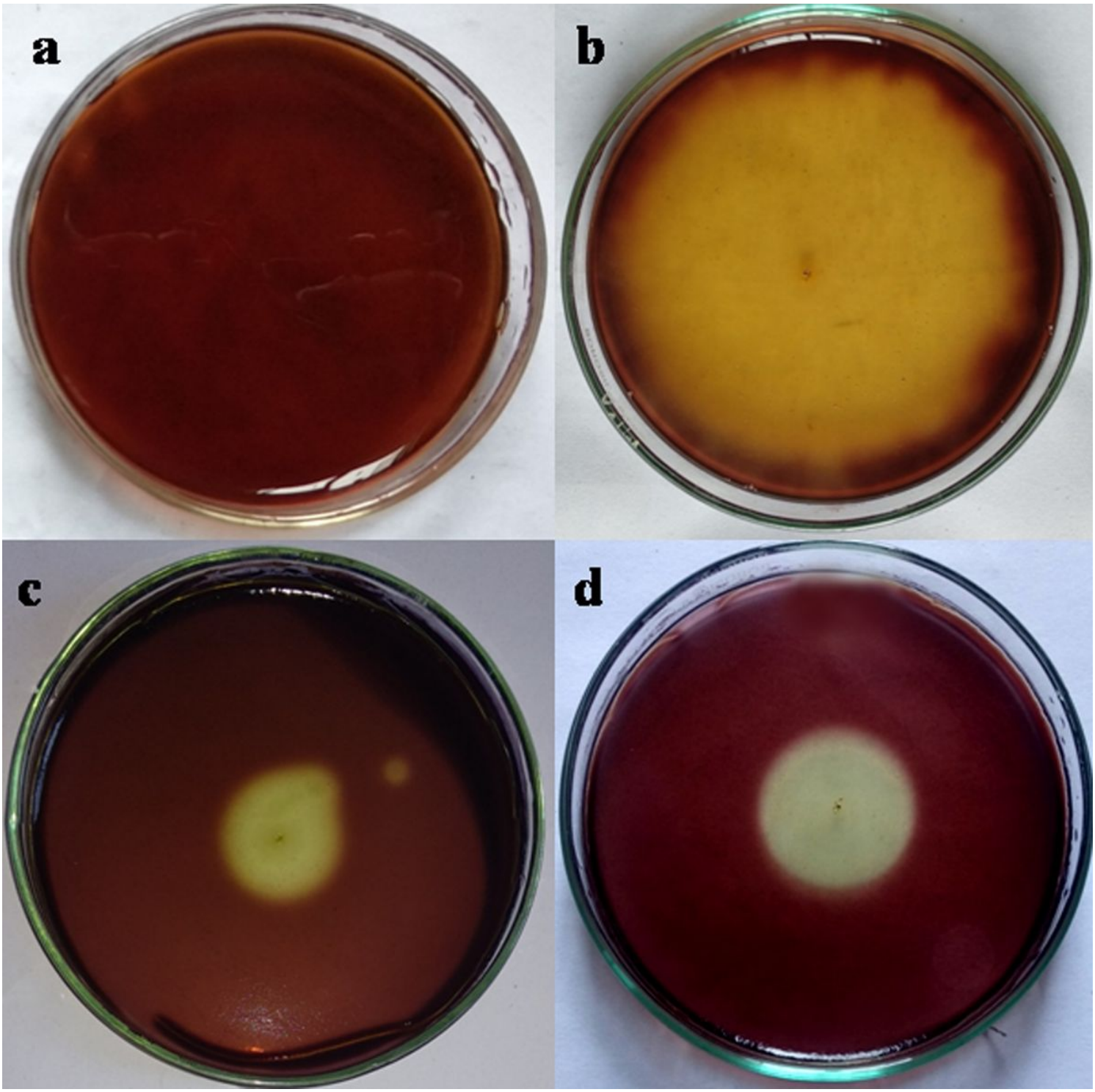

Figure 5

Hydrolytic zone of Chitinase enzyme produced by three fungal isolates in chitin amended agar plates. a. control, b. T. asperellum,c. B. bassiana, d. M. anisopliae.

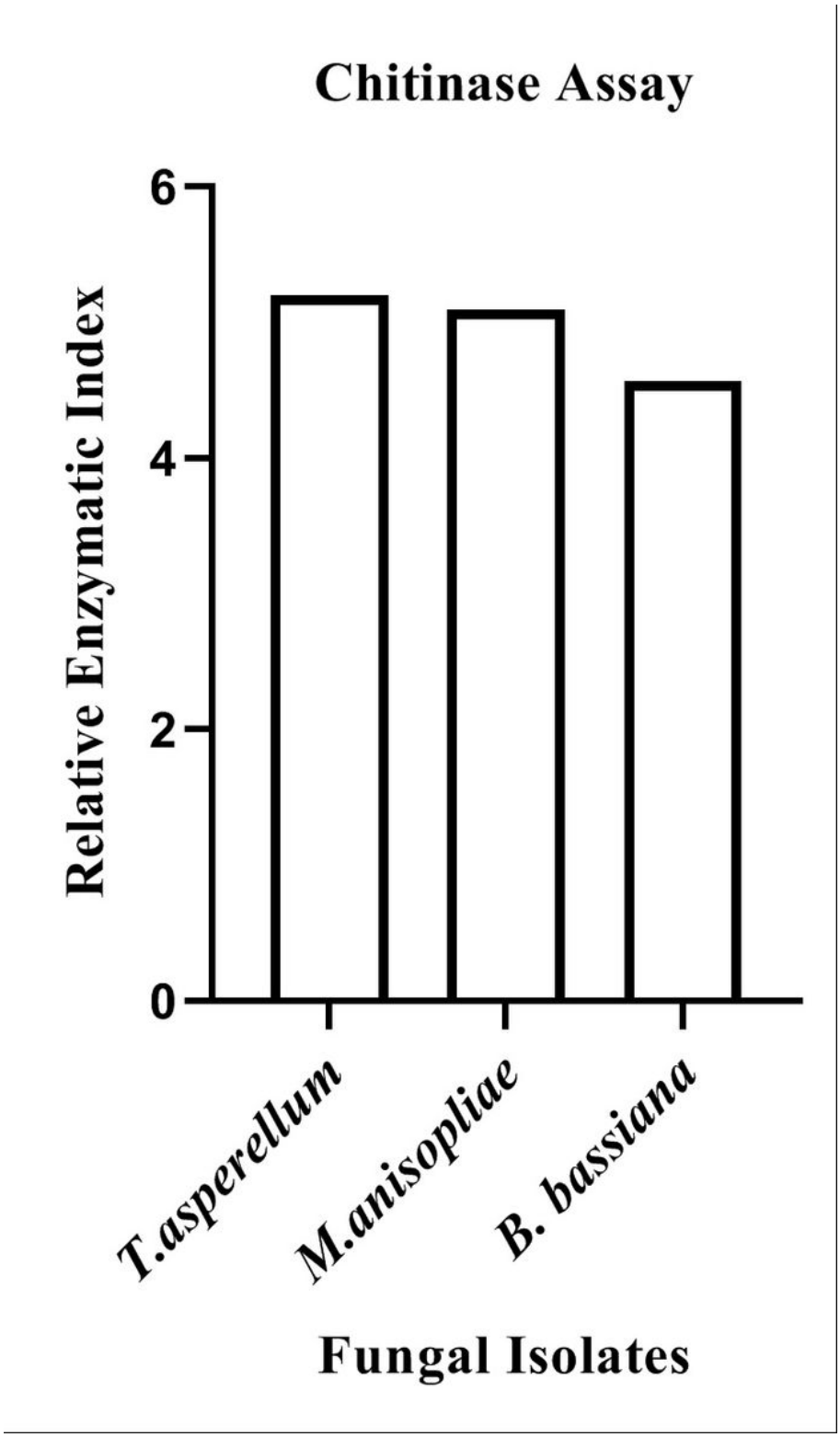

Figure 6

Comparative bar diagram representation of REI value of Chitinase production exhibited by three fungal isolates.

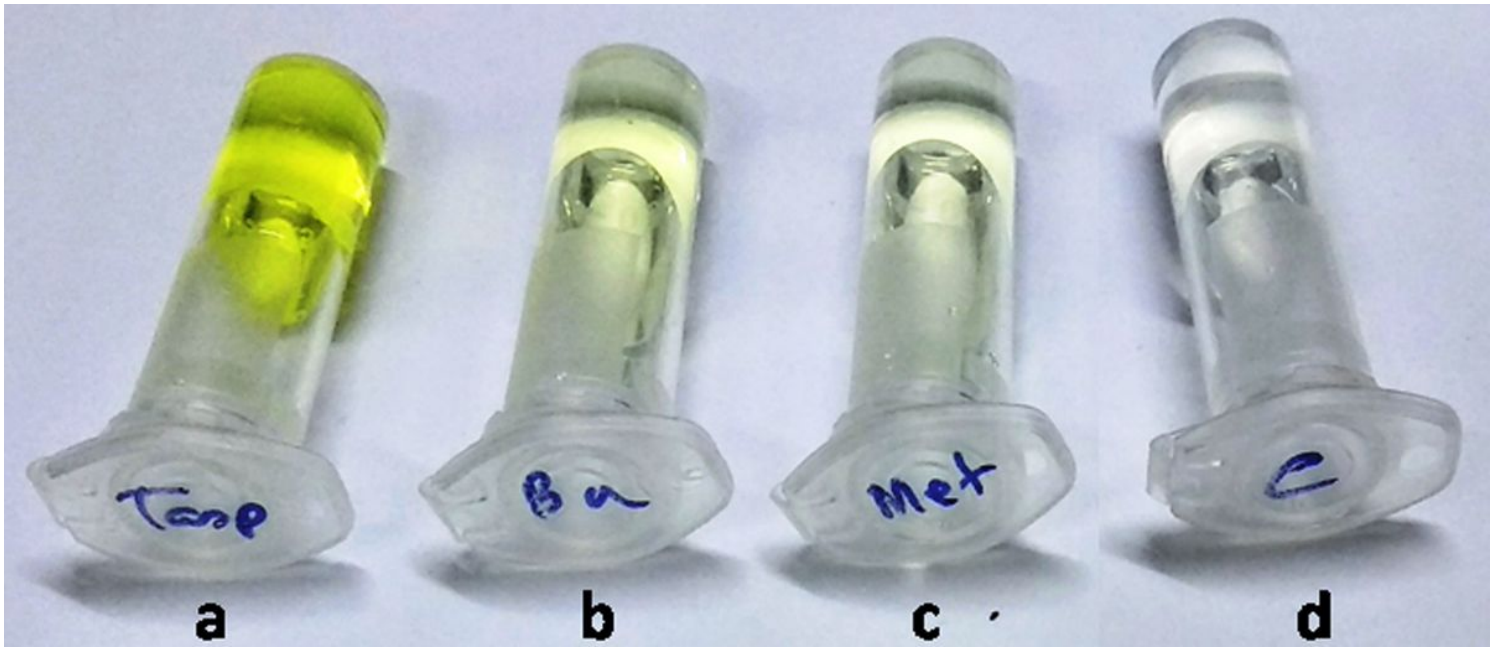

Figure 7

Formation of yellow coloured product (p-nitrophenol) after reaction of PNG with Chitinase enzyme, extracted from culture filtrates of three fungal isolates. a.T. asperellum, b.B. bassiana,c.M. anisopliae, d. control

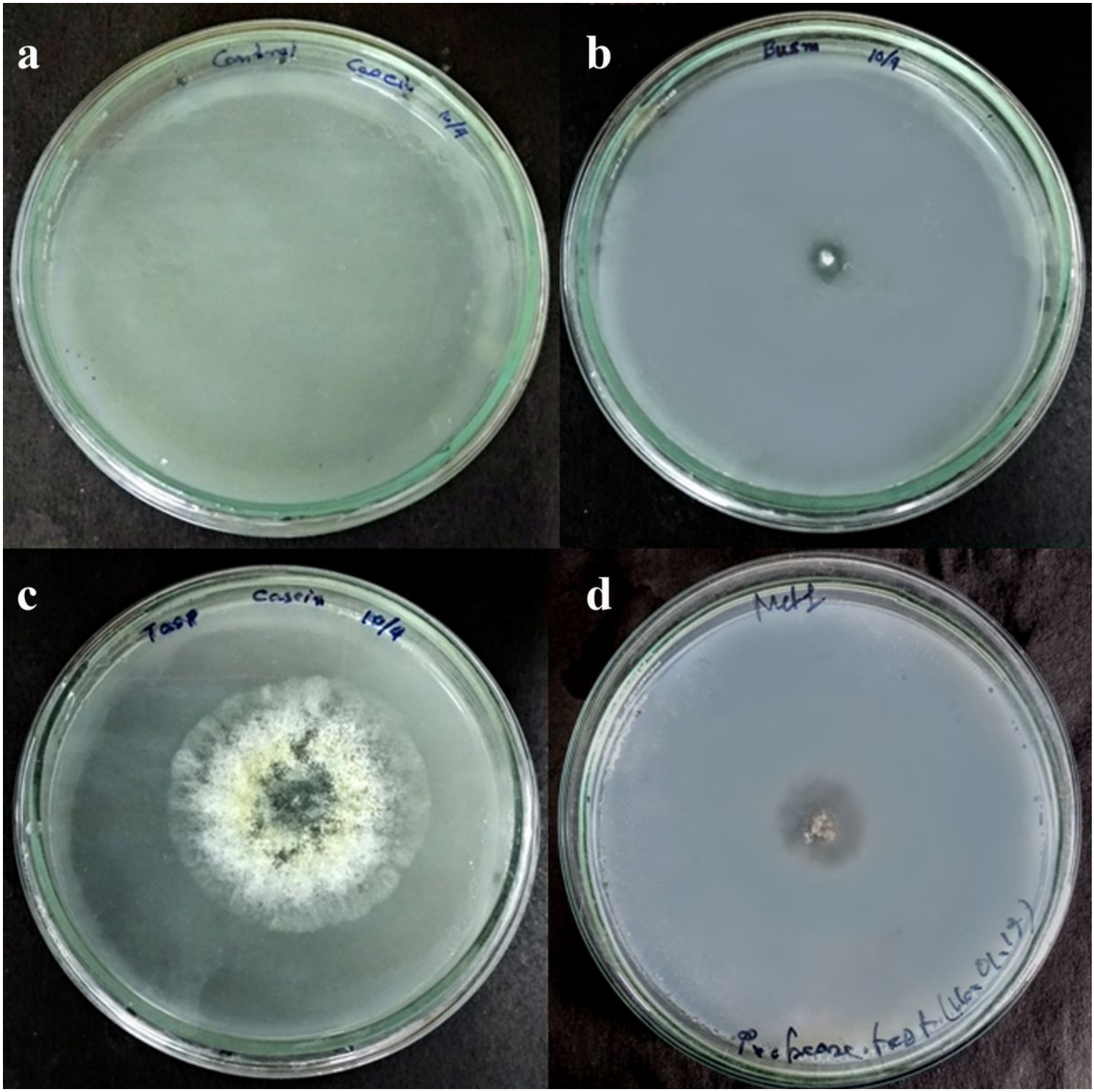

Figure 8

Hydrolytic zone of Protease enzyme produced by three fungal isolates in casein amended agar plates. a. control, b. B. bassiana,c. T. asperellum, d.M. anisopliae.

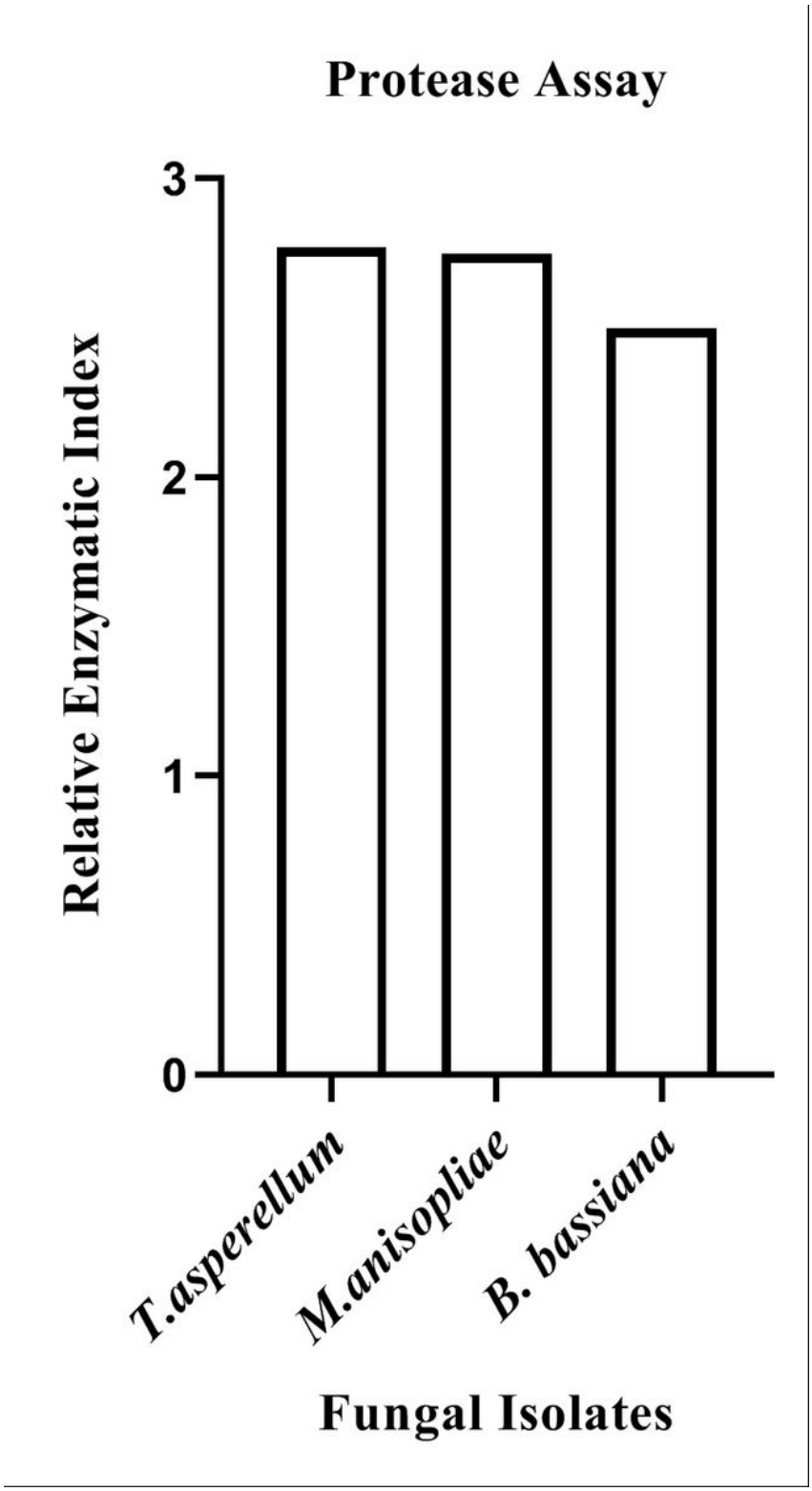

Figure 9

Comparative presentation of REI values of proteases of three entomopathogens in bar diagram

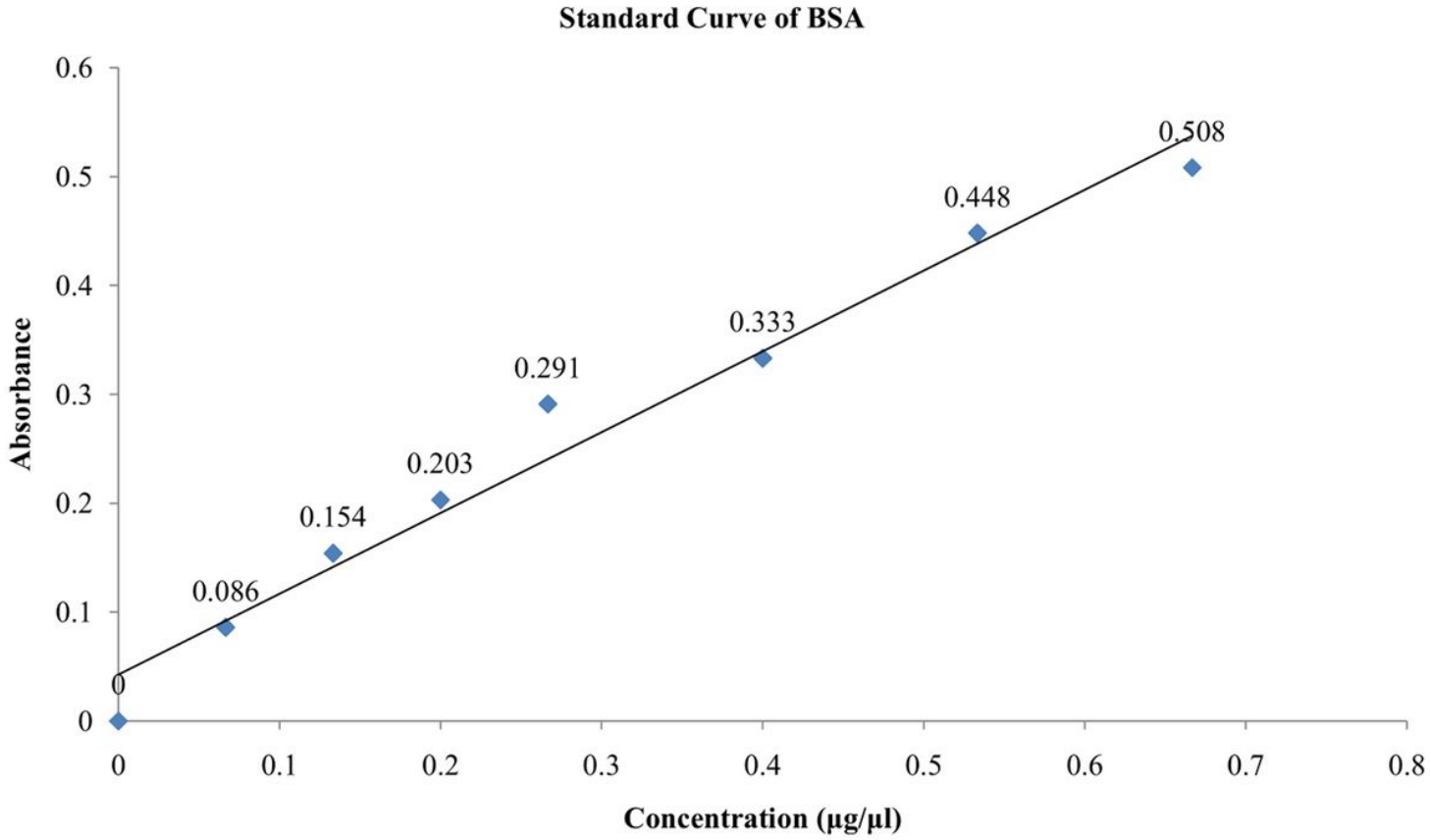

Figure 10

Standard curve of different known concentrations of BSA.

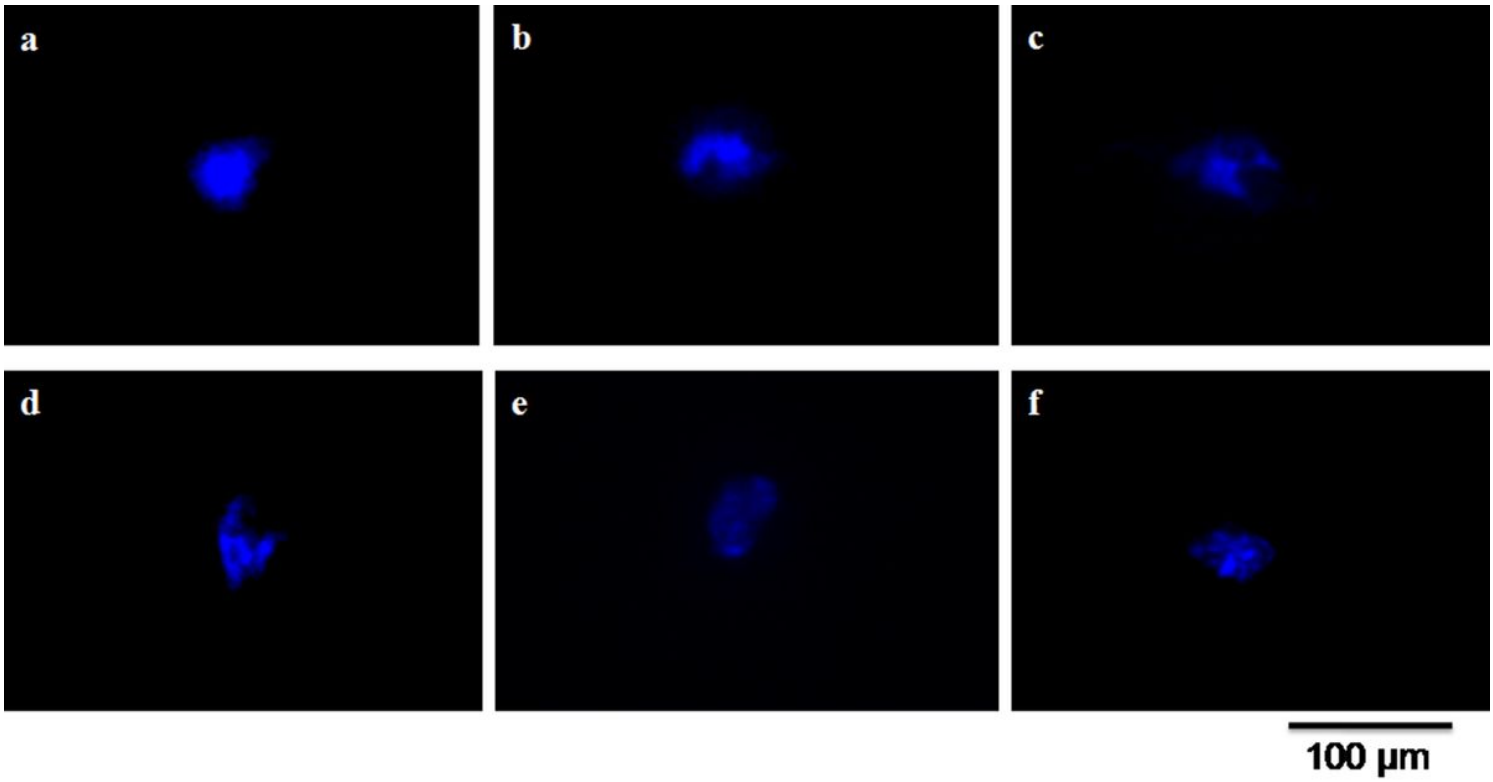

Figure 11

Fluorescent micrographs of nuclear morphologies of larval hemocytes. Hemocytic nucleus of a. non-treated, b. 0.02mg/mL of ME treated, c. 0.04mg/mL of ME treated, d. 0.06mg/mL of ME treated, e. 0.08mg/mL of ME treatedand f. 0.1mg/mL of ME treated larvae.

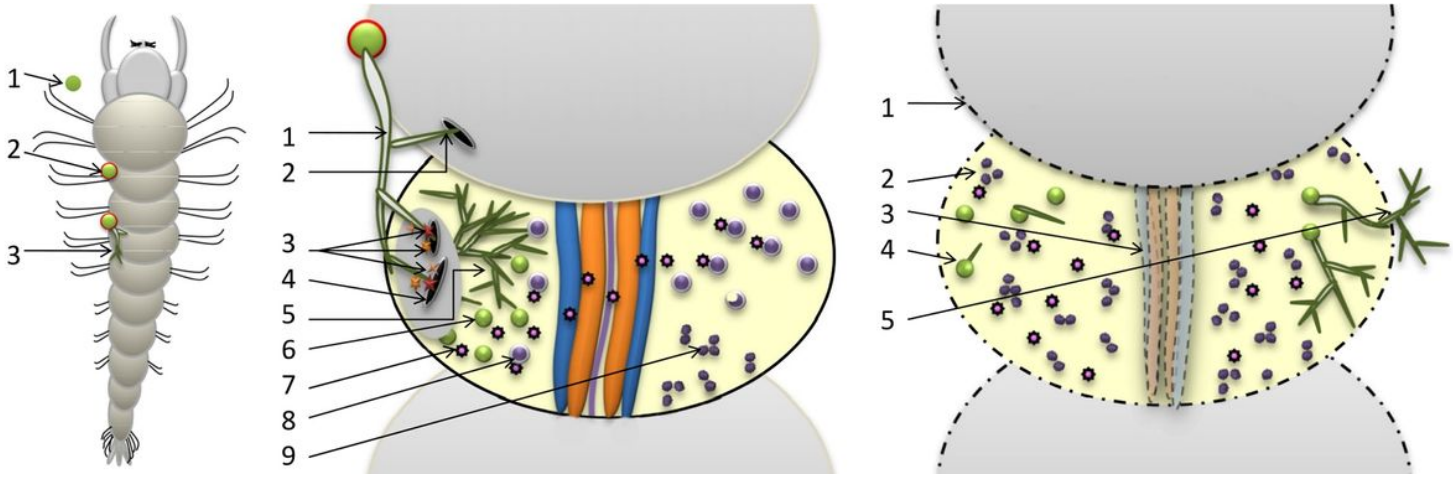

Figure 12

Schematic outline of fungi-larvae interaction. a. Initial stages of fungal interaction, 1. Fungal spore before attachment, 2. Attachment of fungal spore on larval surface by mucilage secretion, 3. Germination of spore on host surface b. Post germination stages of interaction, 1. Appresorrium formation, 2. Formation of infection peg, 3. Secretion of enzymes, 4. Larval cuticle degradation by enzymatic action, 5. Hyphal proliferation, 6. Sporulation, 7. Secretion of toxin, 8. Interaction of toxin with larval hemocyte, 9. Degeneration of hemocyte nucleus c. Final stages of fungal interaction, 1. Degraded segment of dead larvae, 2. Degenerated hemocyte nuclei (immunity breakdown), 3. Shrinkage and deformation of alimentary system (based on information of our earlier work11), 4. Germination of spore inside dead larvae, 5. Proliferation of hyphae outside larval cuticle.