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Development of polyclonal antibody based serological formats for immunodetection of *Metarhizium anisopliae* (Metsch.) Sorokin and *Beauveria bassiana* (Bals.) Vuill.

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Mycelial growth rate of two isolates of *M. anisopliae* and four isolates of *B. bassiana* were compared in four different culture media such as yeast extract agar (YGA), potato dextrose agar (PDA), potato carrot agar (PCA) and beef extract agar (BA). Maximum growth and sporulations were observed in PDA and YGA media, while in BA medium, mycelial growth was found to be very slow even after 20 days of incubation. Among the chosen isolates intra and inter-compatibility tests were performed. Polyclonal antibodies were raised against *Metarhizium anisopliae* isolate 892 and *Beauveria bassiana* isolate 2028 and serological formats such as immunodiffusion, dot blot, PTA-ELISA, Western blot and indirect immunofluorescence were developed for immunodetection of muscardine fungi. Mycelia and conidia of *M. anisopliae* and *B. bassiana* were not autofluorescent nor did they fluoresce when treated with normal serum followed by reaction with FITC. Strong apple green fluorescence was more intense on young hyphal tips of *B. bassiana* and *M. anispliae* when treated with homologous PAb and reacted with FITC, while conidia showed a general fluorescence throughout the surface.

Keywords: *Beauveria bassiana,* dot immunobinding assay, immunodiffusion, indirect immunofluorescence, *Metarhizium anisopliae,* Muscardine fungi

INTRODUCTION

In terrestrial ecosystems decomposition of lignocelluloses is carried out primarily by microorganisms, mainly fungi and bacteria. However, augmenting the activities of microbes is an array of soil macro-invertebrates, whose effects may range from simple dispersion of plant material to actual dissimilation of the structural polymers of lignocelluloses. Among the most abundant and important of these invertebrates are termites, which, with their associated microbial symbionts, dissimilate a significant proportion of the cellulose and hemicelluloses components of the lignocellulosic plant material they ingest. In order to find out the termite species available in and around the Jalpaiguri district, mainly Lataguri forest, Budhaganj forest and some other areas like Danguajhar, Moriabasti, Dewniapara, Domohoni and Kadobari were surveyed. *Odontotermes obesus*, *O. distans*, *O. horni* and *O. boveni* were collected from variable forest plant species.

Extensive damage of wood due to the termite infestation by *Odontotermes obesus* were recorded (Fig.1). Besides, infestation of *Microcerotermes* sp. was also recorded on tea plantation.

There is a resurgence of interest in the use of entomopathogenic fungi for insect pest control. The muscardine fungi *Metarhizium anisopliae* (Metsch.) Sorokin and *Beauveria bassiana* (Bals.) Vuill., are being evaluated worldwide for control of various pests. *Metarhizium anisopliae* strain F52 has been approved as a microbial pesticide active ingredient for non-food use in greenhouses and nurseries. In an environmental risk assessment it was determined that the uses of *Metarhizium anisopliae* strain F52 will have no

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adverse effects on birds, mammals, or terrestrial and aquatic plant species. Combined use of *Metarhizium anisopliae* and entomopathogenic nematodes as a new approach for control of black vine weevil (*Otiorhynchus sulcatus*) has been reported (Ansari *et al.* 2008). Efficacy of *Beauveria bassiana* and *Metarhizium anisopliae* against *Bactrocera cucurbitae* on bitter gourd have been considered by Hamzah *et al*. (2021) as eco-friendly and safer alternative of chemical. Olabiyi *et.al*. (2022) have pointed out that formulated *B. bassiana*-based products applied alone or combined with insect growth regulators (IGR) should be effective tools for managing *Nipaecoccus viridis* populations on young citrus trees protected with mesh exclusion bags. In the present study attempts have been made to raise polyclonal antibody against both the fungi (*M. anisopliae* and *B. bassiana*), develop serological formats for immunodetection of muscardine fungi for their mass production and application in the termite affected field for management.

MATERIALS AND METHODS

Fungal cultures

One isolate of *Metarhizium anisopliae* (MTCC-892) and two isolates of *Beauveria bassiana* (MTCC-984 and MTCC-202) originally isolated from infected *Ips typographus* (Bark Beetle) were collected from Institute of Microbial Technology, Chandigarh, India, whereas another isolate of *B. bassiana* (NCIM-1216) was collected from National collection of Industrial Microorganisms, Biochemical Science Division, National Chemical Laboratory, Pune, India. Besides one isolate each of *M. anisopliae* (BBFF-140) and *B. bassiana* (BBFF-135) were obtained from Biologische Bundersanstalt Fur Landund forstwirtschaft Institut Fur bioloschen ptlanzenschutz Darmstadt, Germany.

For determination of serological cross reactivity against *M. anisopliae* and *B. bassiana,* tea root fungal pathogens such as *Fomes lamaoensis*, *Ustuliza zonata*, *Sphaerostilbe repens* and *Sclerotium rolfsii,* soil borne pathogens viz. *Sclerotinia scleotiorum* and *Fusarium graminearum*; and biocontrol agents viz. *Trichoderma harzianum* and *T. viride* were collected from Immunophytopathology Laboraory, Department of Botany, University of North Bengal. Fungi were grown in Yeast extract glucose agar (YGA) and Potato dextrose agar (PDA) media. These were incubated for 15 d at 28-30^{0}C to allow complete soorulation. All fungal cultures were maintained at room temperature (25-28^{0}C) under weak fluorescent illumination (8 h/day).

Preparation of fungal antigen

Mycelial antigen was prepared following the method as described by Chakraborty *et al*., (1995) Initially the fungal mycelium (4 mm disc) were transferred to 25o ml Ehrlenmeyer flask each containing 50 ml of sterilized liquid yeast extract glucose medium and incubated for 10 days at 28^{0}C. Mycelial mats were washed with 0.2% NaCl and rewashed with sterile distilled water Washed mycelia (50 g fresh wt.) were homogenized with 0.05 M sodium phosphate buffer (pH-7.2) supplemented with 10 mM sodium metabisulphate and 0.05 mM magnesium chloride and 0.85 NaCl in mortar and pestle in the presence of sea sand. Cell homogenates were kept overnight at 4^{0}C. After this period the mixture was centrifuges (15000 rpm) for 30 min at 4^{0}C, the precipitate was dissolved in 10 ml 0.05 M sodium phosphate buffer (pH 7.2). The preparation was dialysed for 72 h through cellulose tubing (Sigma Chemical Co., USA) against 1 L of 0.0005 M sodium phosphate (pH 7.2) with ten changes. Finally the dialysed material was stored at -20^{0}C and used as antigen for production of polyclonal antibody.

SDS-PAGE analysis of total soluble protein

Mycelial protein profile of *M. anisopliae* and *B. bassiana* isolates were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). After electr-ophoresis, the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid:methanol:water (10:20:70) for overnight. The gel was removed from fixer and stained with Coomassie brilliant blue (Sigma R250)staining solution (methanol:glacial acetic :water) for 4 h at 37^{0}C with constant shaking at a very low speed. After staining, the gel was finally destained in methanol:water:acetic acid (4.5:4.5:1) at 37^{0}C with constant shaking until background became clear.

Antisera production and purification of IgG

For the production of antisera against fungal antigens, New Zealand white, male rabbits were used. Before immunization, the body weights of rabbits were recorded and were kept under observation. They were regularly fed with 500 g green grass each time in the morning and evening. Every alternate day they were also given 50-75 g of gram seeds soaked in water. Besides this, they were given saline water after each bleeding for three consecutive days. Normal sera were collected from each rabbit before immunization. Polyclonal antibodies against mycelial antigens of *Metarhizium anisopliae* (isolate 892) and *Beauveria bassiana* (isolate 2028) were raised separately in white male New Zealand rabbits following intramuscular injection of immunogen (1 ml) emulsified with an equal volume of Freund's complete adjuvant (Difco,USA) followed by incomplete adjuvant at weekly intervals, upto 12 weeks. Blood samples were collected by marginal ear vein puncture, 3 days after the first six injections and once in every fortnight, kept at 37^{0}C for 1 h for clotting, followed by centrifugation at 5000 rpm for 10 min at room temperature. IgG was purified from serum using the method of ion exchange chromatography on a DEAE cellulose cloumn as described by Clausen (1988). The concentration of IgG was determined using a standard formula after measuring absorbance for selected fractions at 280 nm and 260 nm.

Agar gel double diffusion test

Agar gel double diffusion test was peformed following the method of Ouchterlony (1967). The antigens and undiluted antisera (50 μl/well) were pipette directly into the appropriate wells and diffusion was allowed to continue in a moist chamber for 48-72 h at 25^{0}C. Precipitation reaction was observed in the ager gel only in cases where common antigens were present. After immunodiffusion, the slides were initially washed with sterile distilled water followed by washing with aqueous NaCl solution (0.9% NaCl mixed with 0.1%Na N_2) for 72 h with 6 hourly changes to remove unreacted antigen and antibody widely dispersed in the agarose. Slides were then stained with Coomassie blue (R250) for 10 min at room temperature. After staining, slides were destained with methanol: water: acetic acid (45:45:10) following changes until the background became clear. Finally, the slides were washed with distilled water and dried in hot air oven for 3 h at 50^{0}C.

Dot immunobinding assay

Dot immunobinding assay was performed using PAbs raised against *M.anisopliae* and *B. bassiana* separately as outlined by Lange *et al*. (1989). Nitrocellulose membrane(NCM- Millipore, H5 SMO 5255, 7cm x 10 cm, pore size 0.45 mm, Millipore Corportaion, Bedford) was first cut carefully into the required size, placed inside Bio-Dot apparatus (Bio-Rad) and 2 ml of coating buffer (carbonate-bicarbonate buffer) was loaded in each well of the template over the NCM and kept for 25 min to dry. Following this 2 ml of mycelial antigens of test samples were loaded into the wells over the NCM and kept for 3 h at room temperature. Template was removed and blocking of NCM was done with 10% non-fat dry milk (casein) prepared in Tris buffer saline (TBS) for 30 min. Polyclonal antibody (IgG – *M. anisopliae* / *B. bassiana* ; 1:40) was added directly in the blocking solution and further incubated at 4^{0}C for overnight. Membrane was then washed several times in TBS-Tween (pH 7.4). Enzymatic reactions were done by treating the NCM with Alkaline phosphatase conjugate (1:7500) for 2 h at 37^{0}C. This was followed by washing for 25 min in TBS-Tween and substrate [66 ml NBT (Nitro Blue Tetrazolium chloride) + 33 ml BCIP (5- Bromo-4-choloro-3-Indolylphophate Di sodium salt) in 10 ml of Tris buffer saline (pH 7.4)] was added. Finally, reaction was stopped by floating the NCM in deionized water.

Plate trapped antigen coated enzyme linked immunosorbent assay (PTA-ELISA)

PTA-ELISA was performed following the method of Chakraborty *et al.* (1995) with modification. Mycelial antigens of *M. anisopliae* / *B. bassiana* were diluted separately with coating buffer (0.05M carbonate bicarbonate buffer, pH 9.6) and the antigens were loaded (200 *M. anisopliae* / *B. bassiana* ml/well) in 8 welled ELISA strips (Coster

EIA/RIA, stripwell plate, USA), arranged in 12 rows in a (cassette) ELISA plate. After loading, the plate was incubated at 25^{0}C for 4 h and washed four times with running tap water and twice with PBS-Tween and each time shaken to dry. Subsequently, 200 ml of blocking reagent (TBS, pH 8.0) was added to each well for blocking the unbound sites and the plate was incubated at 25^{0}C for 1 h and washed again as mentioned earlier. Purified IgG (*M. anisopliae* / *B. bassiana*) was diluted (40mg/ml) separately in antisera dilution buffer (0.15 M PBS-Tween, pH 7.2) and 200 ml loaded in each well and incubated at 4^{0}C overnight.

Fluorescence antibody staining and microscopy

Indirect immunofluorescence staining of mycelia and spores of *M. anisopliae* and *B. bassiana* were done using FITC labeled goat antirabbit IgG following the method of Chakraborty and Saha (1994). Young mycelia (4-day-old) of *M. anisopliae* / *B. bassiana* grown in potato dextrose broth were taken out from the flask and kept in eppendrof tube. Fungal spores of *M. anisopliae* / *B. bassiana* were collected from 15 day-old culture and conidial suspensions prepared with PBS, pH 7.2 were taken in micro-centrifuge tubes and centrifuged at 3000 rpm for 10 min and PBS supernatant was discarded. After washing mycelia with PBS (phosphate buffer saline), pH7.2 and spores were separately treated either with normal sera or antisera diluted (1:125) with PBS, pH 7.2, incubated for 30 min at 27^{0}C, washed thrice with PBS-Tween (pH 7.2) and treated with goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma) diluted with PBS (1:40) and incubated in dark for 45 min at room temperature. After incubation specimen were washed thrice and mounted in 10% glycerol. Then slides were observed using a Biomed microscope (Leitz) equipeed with an I-3 filter block ideal FITC fluorescence under UV light in dark. Photographs were raken by Moticam Pro 285B.

Western blotting

SDS-PAGE of mycelial antigen was carried out in a mini gel unit. Following gel run, it was transferred to Towbin buffer and equilibrated for 1 h and blot transfer was done on NCM. Blocking of NCM was done with 10% non-fat dry milk (casein) prepared in Tris buffer saline (TBS) for 30 min. IgG of *M. anisopliae* (1:40) was added directly in the blocking solution, incubated in plastic bag at 4^{0}C for overnight and membrane was then washed several times in TBS-Tween (pH 7.4). Enzymatic reactions were done by treating the NCM with Alkaline phosphatase conjugate (1:10,00) for 2 h at room temperature. This was followed by washing for 25 min in TBS-Tween and substrate (66 ml NBT + 33 ml BCIP in 10 ml of Tris buffer saline, pH 7.4) was added. The reaction was monitored carefully. On visualization of the bands up to the desired intensity the membrane was transferred to a tray flooded with stop solution (Wakeham and White, 1996).

RESULTS

Evaluation of mycelial growth and sporulation of M. aniospliae and B. bassiana isolates

Mycelial growth rate of two isolates of *M. anisopliae* and four isolates of *B. bassiana* were observed initially in four different culture media such as yeast extract agar (YGA), potato dextrose agar (PDA), potato carrot agar (PCA) and beef extract agar (BA). Maximum growth and sporulations were observed in PDA and YGA media (Table 1, Fig. 1), while in BA medium mycelia growth was found to be very slow even after 20 days of incubation. On this basis YGA and PDA were selected for spore production. Standardization of conidial concentration were done with 28 days old cultures when maximum spore production was noticed. All the isolates of *M. anisopliae* and *B. bassiana* were grown in Potato dextrose broth (PDB) for 28 days and their conidial concentrations were determined from the serial dilution of the stock suspension. Highest conidial concentration (4.56 X 10^6 conidia/ml) at 10^{-1} dilution was recorded for *B. bassiana* isolate 2028, while *M. anisopliae* isolate 140 registered lowest conidial concentration (2.3 X 10^6 conidia/ml) at same dilution (Table 2).

Compatibility tests for M. aniospliae and B. bassiana isolates

Among the chosen isolates intra and inter-compatibility tests were performed. The results

Fig.1. Termite infestation and damaged (A) Teak and (B) Tea plants; (C) Underground termite nests

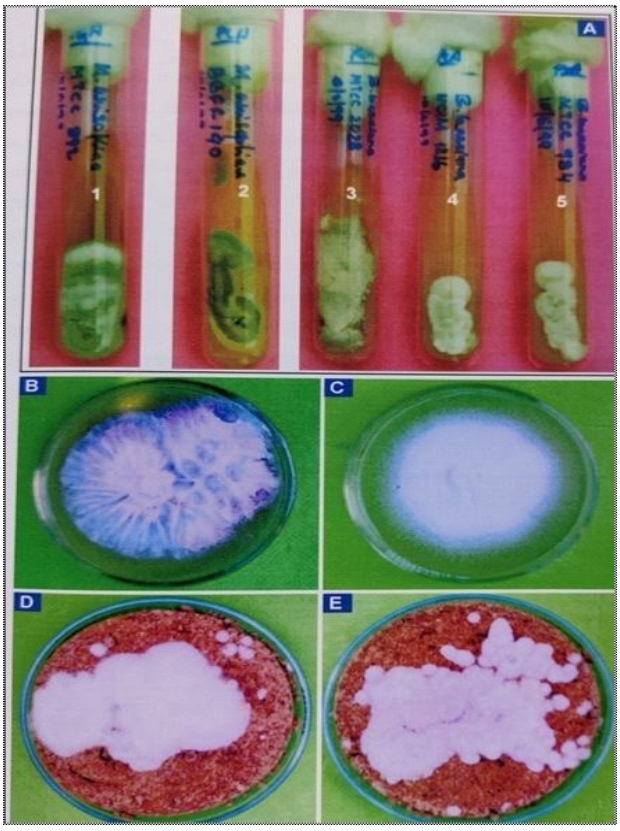

Fig. 2: (A) Cultures of *Metarhizium anisopliae* (A1-892, A2-140) and *Beauveria bassiana* (A3-2023, A4-1216, A5-984). Mycelial growth of *M. aniosopliae* isolate 892 (B&D)*B. bassiana* isolate – 2028 (C&E) in PDA (B&C) and maize meal (D&E) media

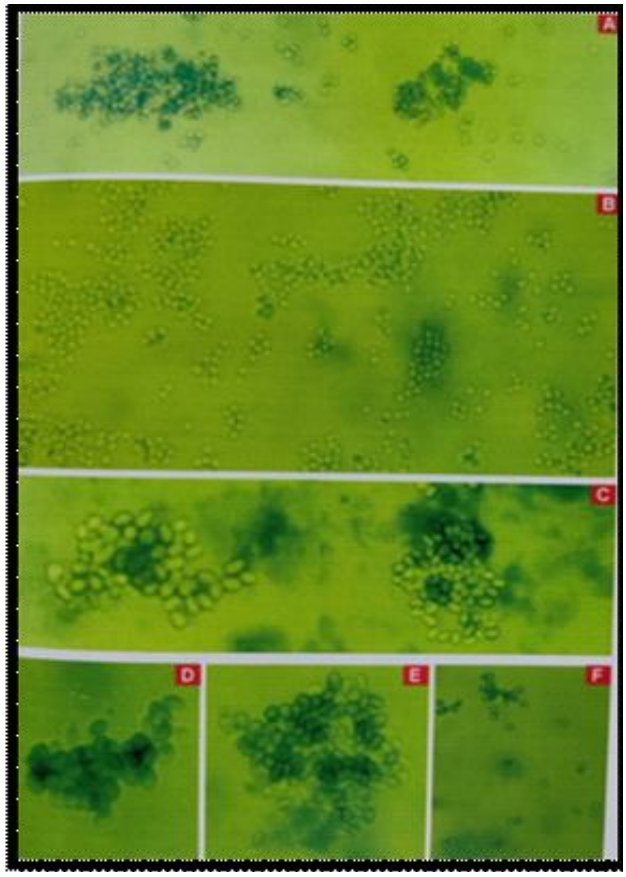

Fig. 3 Conidia of *Metarhizium anisopliae* isolates 892 (A&F), 140 (B) and *Beauveria bassiana* isolates 2028 (C),1216 (D) and 984 (E)

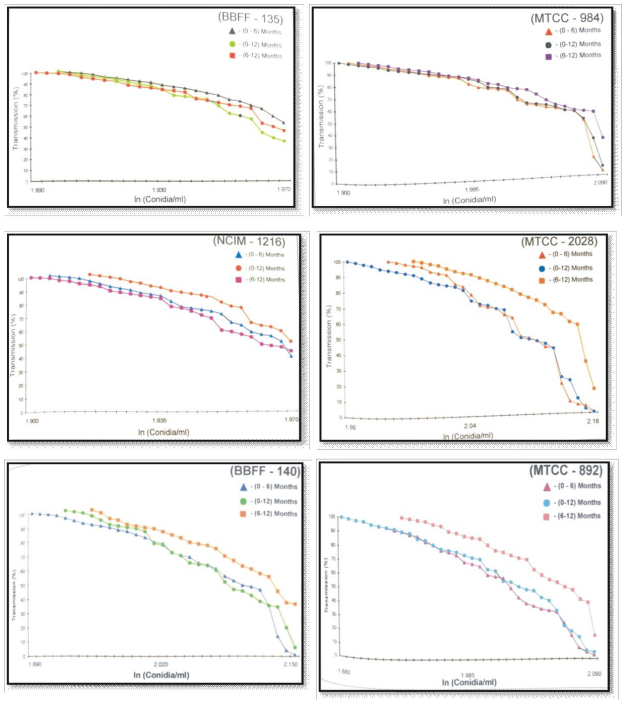

Fig. 4: Transmission (%) and their corresponding conidial count for isolates of *Beauveria bassiana* – BBFF-135, MTCC-984, NCIM-1216, MTCC-2028 and *Metarhizium anisopliae* – BBFF-140, MTCC-892 cultured in different periods.

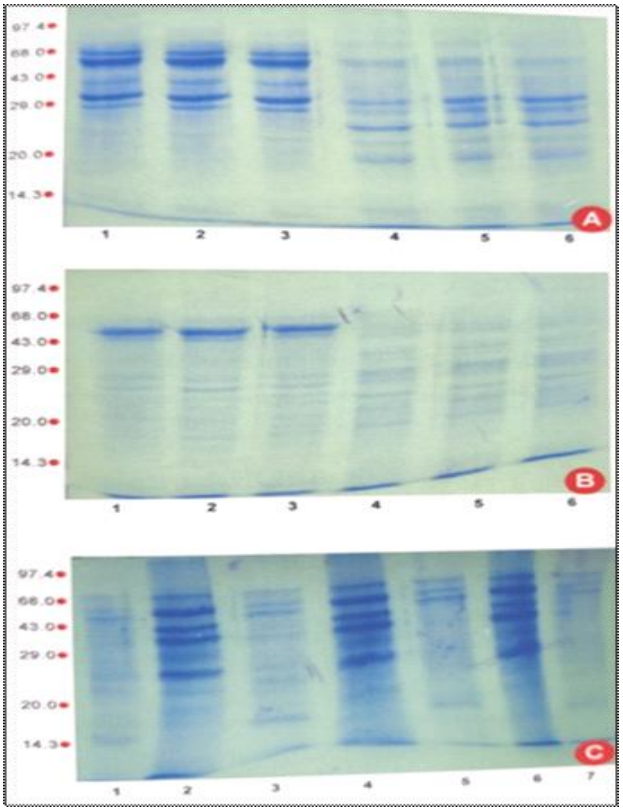

Fig.5. SDS-PAGE analysis of mycelia protein. (A) *M. anisopliae* isolate-892 (Lanes 1-3) and *B. bassiana* isolate-2028 (Lanes 4-6); (B) *B. basisiana* isolates -1216 (Lanes 1-3), 984 (Lanes 4-6); (C) *B. bassiana* isolate 135 (Lanes 1,3,5 & 7) and *M. anisopliae* isolate- 140(Lanes 2,4 & 6)

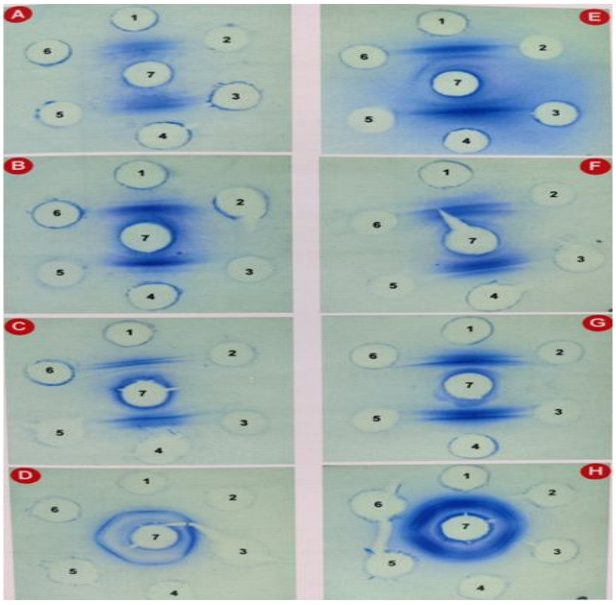

Fig 6. Agar gel double diffusion tests with PAbs of (A-D) *M. aniospliae* isolate 892 and (E-H) *B. bassiana* isolate 2028. Peripheral wells were loaded with mycelial antigens of *M. anisopliae* isolates 892 (wells 1&4); 140 (wells 2&5) and *B. bassiana* isolates 135 (well 3); 2028 (well 6). Central wells were loaded with PAb. [A&E – 1st bleeding, F-2nd bleeding, B&G – 3rd bleeding and C,D&H – 4th bleeding]

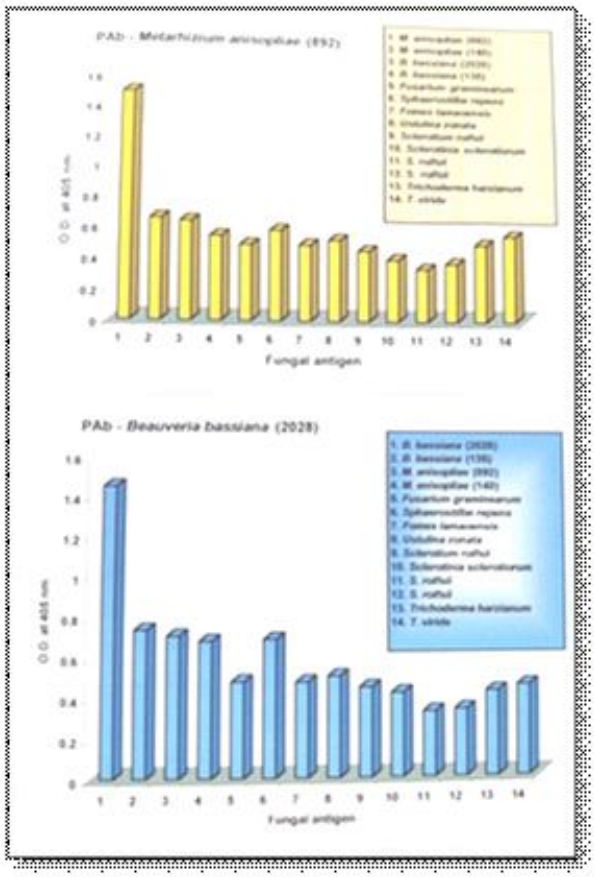

Fig.7. Serological cross reactivity of PAbs of *M. aniosopliae* (892) and *B. bassiana* (2028) with other fungi using PTA-ELISA format

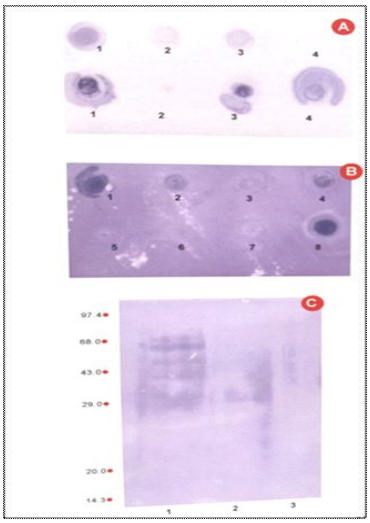

Fig. 8. (A&B) Dot immunobinding assay using mycelia antigens and PAbs of (A) *M. anisopliae* and (B) *B. bassiana* reacting with mycelial antigens of *B. bassiana* isolates 2028 (1), 1216 (2), 135 (3) and 984(4); *M. anisopliae* isolates 892(5),140(6), soil amended antigens of two isolates - Ma 892(7) and Bb2028 (8).**(C)** Western blot analysis of mycelia antigens of *M. anisopliae* islate 892(lane1), soil amended antigens of *M. anisopliae* isolate 892 (lane 2) and mycelia antigen of *M. anisopliae* isolate 140 (lane 3). The blot was probed with PAb prepared from mycelia antigen of *M. anisopliae* isolate 892 on nitrocellulose paper.

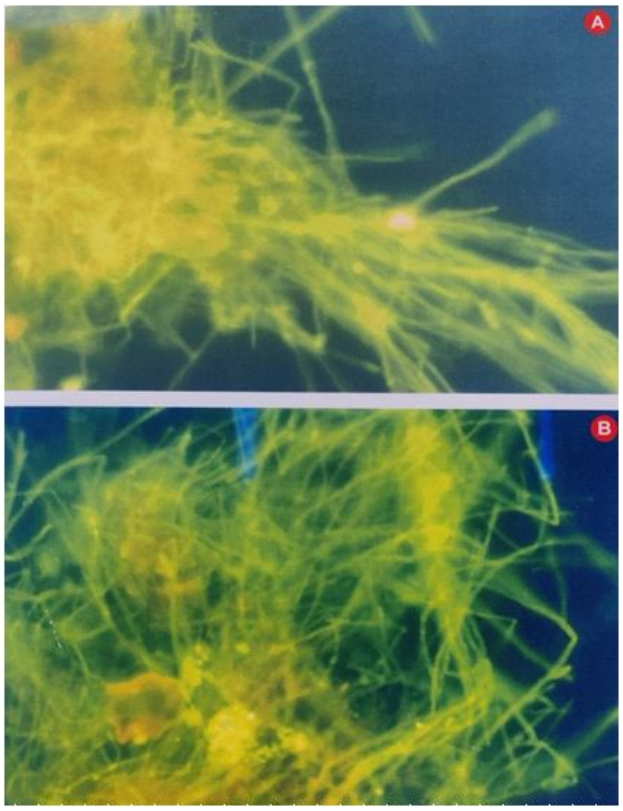

Fig.9: Indirect immunofluorescence of hyphae of *M. aniospliae* isolate 892 (A) and *B. bassiana* isolate 2028 (B) treated with homologous PAB and reacted with FITC lebelled antibodies of goat specific for rabbit globulin

of compatibility tests for isolates of *M. aniosopliae* and *B. bassiana* are given in Table 2. Excellent growth was observed among the isolates of B. bassiana; Bb-2028 and Bb -1216; Bb-1216 and Bb-984 and Bb-984; and between *B. bassiana* (135) and *M. anisopliae* (140). The growth was at medium rate in the combinations of (a) Bb-2028 and Bb-135; (b) Bb-1216 and Bb-135; (c) Bb-984 and Bb-135. Good form of growth was obtained in the combinations of Bb-2028 and Bb-984; Bb-2028 and Ma-892; Bb-121 and Ma-892; Bb-1216 and Ma-140; Bb-984 and Ma -892; Bb-135 and Ma -892 and Ma-892 and Ma-140 (Table 3).

Determination of conidial concentration of fungal isolates using transmission measurements

Four isolates each of *B. bassiana* and two isolates of *M. anisopliae* were used for haemocytometer counts and transmission measurements from 28 day old cultures for a period of 12 months. Bright field microscopic observations of the conidia have

Table. 1: Mycelial growth of *B. bassiana* and *M. anisopliae* isolates in different culture media

	[a]Average mycelial growth in different media (cm)			
Fungal isolates	YGA	PDA	PCA	BA
B. bassiana (135)	5.2	7.7	3.9	2.5
B. bassiana (984)	5.4	6.8	2.5	1.8
B. bassiana (2028)	7.2	7.1	3.2	1.3
B. bassiana (1216)	7.0	7.8	2.8	1.5
M. anisopliae (140)	7.2	8.2	4.2	1.2
M. anisopliae (892)	7.3	8.1	4.4	2.5

Incubation period – 20 days, Temperature – 28^{0}C
[a] Average of five replicates
YGA = Yeast extract Glucose agar ; PDA = Potato dextrose agar; PCA = Potato Carrot agar; BA = Beef extract agar

Table 2. Conidial concentrations of *B. bassiana* and *M. aniopliae* isolates at different serial dilutions

Isolates	'O' suspension	Serial dilutions			
		10^{-1}	10^{-2}	10^{-3}	10^{-4}
Bb-2028	$5.2X10^7$(12)	$4.6X10^6$(13)	$9.9X10^5$(15)	$2.7X10^5$(14)	$5.4X10^4$(12)
Bb-1216	$2.9X10^7$(10)	$3.6X10^6$(11)	$8.9X10^5$(11)	$2.4X10^5$(11)	$5.9X10^4$(11)
Bb-984	$4.0X10^7$(14)	$3.8X10^6$(14)	$8.7X10^5$(16)	$2.5X10^5$(16)	$8.4X10^4$(14)
Bb-135	$3.0X10^7$(8)	$3.3X10^6$(8)	$7.3X10^5$(7)	$2.7X10^5$(7)	$6.8X10^4$(7)
Ma-892	$3.0X10^7$(18)	$3.3X10^6$(18)	$7.2X10^5$(22)	$2.7X10^5$(20)	$6.1X10^4$(18)
Ma-140	$2.9X10^7$(18)	$2.3X10^6$(18)	$3.2X10^5$(18)	$1.2X10^5$(18)	$3.6X10^4$(18)

Figures in parenthesis are the number of replications. Bb – *Baeuveria bassiana* ; Ma – *Metarhizium anisopliae* Conidial concentrations were determined by haemocytometer measurements of each dilutions

Table 3 : Intra- and Inter-compatibility test among the chosen isolates of *B. bassiana* and *M.anisopliae*

Combinations	Growth performance[a]
1 and 2	++++
1 and 3	+++
1 and 4	++
1 and 5	+++
1 and 6	++
2 and 3	++++
2 and 4	++
2 and 5	+++
2 and 6	+++
3 and 4	++
3 and 5	+++
3 and 6	++
4 and 5	+++
4 and 6	++++
5 and 6	+++

1 = Bb 2028; 2 = Bb 1216; 3 = Bb 984; 4 = Bb 135; 5 = Ma 892; 6 = Ma 140 [a]After 10 days observation with three replicates
++ = Medium ; +++ = Good; ++++ = Excellent

Table 4. Regression relationship between numbers of conidia (X) in suspension and percentage transmission (Y) at different periods for isolates of *M. anisopliae* and *B. bassiana*

Isolates	Regression relationship[a]
M. anisopliae BBFF-140	X=1.95+(-0.004) Y Y= 87.68+(-18.5) X
M. anisopliae MTCC -892	X=1.96+(-0.0006) Y Y= 80.74+(-14.5) X
B. bassiana NCIM - 1216	X=2.00+(-0.001) Y Y= 87.05+(-16.75) X
B. bassiana MTCC-2028	X=4.87+(-0.057) Y Y= 646.27+(-303.68) X
B. bassiana BBFF-135	X=1.99+(-0.001) Y Y= 166.53+(-55) X
B. bassiana MTCC -984	X=1.98+(-0.0008) Y Y= 64.7+(-4) X

[a]Regression line for *M. anisopliae* and *B . bassiana* isolates for the relationship between transmission (%Y) and numbers of conidia in suspension (X) are based on 15 selected data points at different periods of 0-6, 0-12 and 6-12 months nearest to 50% transmission

been presented in Fig.3. For each of the six isolates of *M. anisopliae* and *B. bassiana*, three lines, representing the measurements on the different chosen periods (0-6, 0-12 and 6-12 months) have been presented in Fig 4 . The regression relationship between the number of conidia in suspension (X) and percentage transmission (Y) at different periods of 0-6, 0-12 and 6-12 months for different isolates of *M. aniopliae* and *B. bassiana* have been mentioned in Table 4.

SDS-PAGE analysis of fungal isolates

SDS-PAGE was performed for the detailed analysis of the protein profile of different isolates of *M. aniopliae* and *B. bassiana.* On the gels, coomassie blue stained band were observed for the mycelial proteins of the isolates (Fig. 5). In *M. anisopliae* isolate 892 seven bands were observed and their molecular weight ranged from 72.6 – 22.4 KDa (Table 5). In *M. anisopliae* isolate 140, ten bands were recorded and the molecular weight ranged from 83.6 – 18.4. In B. bassiana isolate 2028, 984 and 135 the number of protein bands recorded were 14, 11, 12 and 11 respectively. Their molecular weights have been depicted in Table 5.

Immunodiffusion test

The effectiveness of antigen preparation from the mycelia of *M. anisopliae* (isolate 892) and *B. bassiana* (isolate 2028) in raising antibodies was checked by homologous cross reaction following agar gel double diffusion technique. Strong precipitin reactions were observed when antiserum raised against mycelia of *M. anisopliae* (isolate 892) and *B. bassiana* (isolate 2028) was reacted against its own antigen and weak or no precipitin reaction were observed with antigen of other isolate (Fig 6, Table 6)

Optimization of antigen and PAb concentrations using PTA –ELISA formats

Polyclonal antibodies (PAbs) were raised against mycelial antigens of *M. aniosopliae* and *B. bassiana.* Optimization of PAbs concentration through PTA-ELISA was done considering two variables i.e. concentration of PAb (IgG) and concentration of mycelia antigens. A series of dilution from 10,000 ng to 625 ng of mycelial antigens of *M. anisopliae* (isolate 892) and *B. bassiana* (isolate 2028) were made and tested separately against two antisera dilutions (1:125 and 1:250). Results (Table 7) revealed that ELISA values decreased with increasing dilution of antigen. The absorbance values of 1:125 antiserum dilution was relatively higher then 1:250 antiserum dilution.

Determination of serological cross reactivity of PAbs of M. aniosopliae and B. bassiana

Cross reactivity of the PAbs raised against *M. anisopliae* (892) and *B. bassiana* (2028) were tested against mycelia antigens of two isolates each of *M. anisopliae* (892 &140) and *B. bassiana* (2028 & 135), tea root fungal pathogens such as *Fomes lamaoensis*, *Ustuliza zonata*, *Sphaerostilbe repens* and *Sclerotium rolfsii,* soil borne pathogens viz. *Sclerotinia scleotiorum* and *Fusarium graminearum*; and biocontrol agents viz. *Trichoderma harzianum* and *T. viride* using PTA-ELISA format. Results (Fig. 7) revealed that in comparison with highest absrbance in homologous reaction (Ma892 & Bb2028), other isolates of *M. aniospoliae* (140) and *B. bassiana* (135) also showed maximum absorbance along

Table 5 : Molecular weights of soluble mycelia proteins of *M. aniospliae* and *B. bassiana* isolates resolved on SDS-PAGE

Fungal isolates	Number of protein bands[a]	Molecular weight (KDa)
M. anisopliae MTCC -892	7	72.6, 59, 50, 37.4, 29.4, 28.2,22.4
M. anisopliae BBFF-140	10	83.6, 79.4, 68, 59, 54, 40.8, 29.4, 26.6, 19.6, 18.4
B. bassiana MTCC-2028	14	54.5, 50, 34.5, 29.8, 29.4, 26.1, 25.4, 24.5, 23.2, 22.4, 21.6, 19.6, 17.7, 15.8
B. bassiana NCIM - 1216	11	54.2,50,47.5,43, 28.2,26.6, 26, 23.2, 22.4, 19.6, 17.6
B. bassiana MTCC -984	12	59,50, 43, 37.4, 29.8, 29.4, 29, 28.2, 26, 24.2, 20.8, 17.7
B. bassiana BBFF-135	11	68, 59,50, 47.4, 38.6, 34.5, 24.5, 21.6, 18.4, 16.5, 14.8

[a] Coomassie blue stained mycelia protein bands

Table 6. Detection of cross reactive antigens among different isolates of *M anisopliae* and *B. bassiana* in immunodiffusion test

Mycelial antigen	PAb of *M. anisopliae* (isolate 892)	PAb of *B. bassiana* (isolate 2028)
M. anisopliae MTCC -892	**++++**	**-**
M. anisopliae BBFF-140	**+**	**-**
B. bassiana BBFF-135	**-**	**+**
B. bassiana MTCC-2028	**-**	**++++**

(++++) Strong precipitin bands; (+) Weak precipitin band ; (-) Common precipitin band absent

Table 7. Optimization of mycelia antigen and PAb concentrations of *M. anisopliae* (892) and *B. bassiana* (2028) using PTA –ELISA formats

	M. anisoliae(isolate 892)		*B. bassiana* (isolate 2028)	
	Absorbance at 405 nm			
Antigen concencentration (ng/ml)	Antiserum dilution 1:125	1:250	Antiserum dilution 1:125	1:250
10,000	1.293	1.232	1.317	1.252
5,000	1.287	1.142	1.239	1.228
2,500	1.229	0.847	1.212	1.218
1,250	1.029	0.734	1.136	1.069
625	1.095	0.672	0.957	1.011

Table 8. Dot blot reaction of PAbs raised against *M. anisopliae*(892) and *B. bassiana* (2028) with mycelial antigens of other isolates of *M. anisopliae* and *B. bassiana*

	Colour intensity	
Mycelial antigen	PAb of *M. anisopliae* (892)	PAb of *B. bassiana* (2028)
Ma 892	++++	+++
*Ma*140	+	++
*Ma*892 (amended soil)[a]	++++	++
*Bb*2028	+++	++++
*Bb*1216	++	++
*Bb*135	++	+
*Bb*984	-	+
*Bb*2028(amended soil)[a]	+++	+++

[a] Antigen prepared from soil amended with spores and mycelia
Colour intensity: Bright (++++), High (+++), Low (++), Faint (+) and no colour (-)

with *Sphaerostilbe repens*. Minimum homology was exhibited by antigen of *Sclerotium rolfsii*.

Dot immunobinding assay and western blot analysis

Dot immunobinding assay was performed on nitrocellulose membrane using PAb of *M. anisopliae* (Fig. 8A) and *B. bassiana* (Fig. 8B) reacting with mycelial antigens of *B. bassiana* isolates 2028, 1216, 135 and 984; *M. anisopliae* isolates 892,140, soil amended antigens of two isolates (Ma 892 & Bb2028). Colour intensity of dot blots have been presented in Table 8.

Western blot analysis of different antigens following SDS-PAGE were performed probing with PAb of *M. anisopliae* isolate 892. Homologous antigen exhibited six bands with molecular weights between 55-16 KDa, while amended soil antigen of *M. anisopliae* (892) revealed four bands with molecular weights between 37.5 – 21 KDa (Fig 8C).

Indirect immunofluorescence study of mycelia of M. anisopliae and B. bassiana

Mycelia and conidia of *M. anisopliae* isolate 892 and *B. bassiana* isolate 2028 were not autofluorescent nor did they fluoresce when treated with normal serum followed by reaction with FITC. Treatment of mycelia of *M. anisopliae* isolate 892 with homologous PAb and reacted with FITC showed bright apple green fluorescence (Fig.9A). Strong apple green fluorescence was more intense on young hyphal tips of *B. bassiana* (Fig. 9B) when treated with homologous PAb and reacted with FITC, while conidia showed a general fluorescence throughout the surface.

DISCUSSION

Metarhizium anisopliae and *Beauveria bassiana* are widespread entomopathogenic fungi. Their high pathogenicity to termites have already been recognized (Ansari *et al.* 2008) and their suitability in pest control has been verified. Biocontrol competence of *Beauveria bassiana*, *Metarhizium anisopliae* and *Bacillus thuringiensis* against tomato leaf miner, *Tuta absoluta* Meyrick 1917 under greenhouse and field conditions have been reported (Aynalem *et.al*. 2022). Suitability of formulated entomopathogenic fungi against *Hibiscus* mealybug, *Nipaecoccus viridis*, deployed within mesh covers intended to protect Citrus from Huanglongbing have been discussed by Olabiyi *et.al*. (2022).They produce two types of spores, basidiospores in the living host and conidia on the surface of the dead host. Artificial culturing of the isolates of *M. aniospliae* and *B. bassiana* , their growth and conidial concentrations have been determined. A utlizable source of carbon for germination, a nitrogen source for continuous hyphal growth, high humidity (80-100% R.H.), and optimum temperature between 25^0C – 28^0C are necessary. For molecular characterization of the entomopathogenic fungi antigens were prepared from mycelia and conidia of all the isolates of *M. aniospliae* and *B. bassiana*, resolved in SDS-PAGE and finally polyclonal antibodies (PAb) were generated against *M. anisopliae* isolate 892 and *B. bassiana* isolate 2028. Initially, effectiveness of PAbs raised against *M. anisopliae* and *B. bassiana* was confirmed by immunodiffusion. Optimization of PTA-ELISA was done considering two variables – dilution of antigen and antiserum. The results of PTA ELISA were confirmed by dot-immunobinding assay (DIBA) in which intensity of the dots were compared for homologous and heterologous cross reactions. A good correlation was found between the two techniques as results of DIBA gave similar level of detection as in PTA ELISA.

In the present investigation, cross reactivity of PAbs raised against *M. anisopliae* and *B. bassiana* were tested against other soil fungi, such as *Fusarium graminearum*, *Sphaerostilbe repens, Fomes lamaoensis, Sclerotium rolfsii, Sclerotinia sclerotiorum, Trichoderma harzianum* and *Trichoderma viride* using PTA-ELISA formats. Positive reactions in ELISA with higher absorbance were always evident from the homologous fungal antigens of both the entomopathogenic fungi (Ma892 & Bb2028). Other isolates of *M. aniospoliae* (140) and *B. bassiana* (135) also showed maximum absorbance along with *Sphaerostilbe repens*. Minimum homology was exhibited by antigen of *Sclerotium rolfsii*. Therefore, PTA-ELISA format has been fund sensitive to detect *M. anisopliae* and *B. bassianan* on termites following application as biocontrol. It could overcome difficulty observed in visually detecting slight infection of

termites in their colony. The ELISA test might also prove useful in monitoring field collected samples of entomopathogens for identification and ensure their infection potential. Molecular probing of soil antigen amended with mycelia and conidia of entomopathogenic fungi was also performed with PAb raised against mycelial antigen of *M. anisopliae*(892). This western blot analysis also helped us to investigate the persistence and to fix up spray schedule of the entomopathogen(s) in soil following field application of the formulated mycoinsecticide.

Treatment of mycelia and conidia of *M anisopliae* and *B. bassiana* with homologous PAb and reacted with FITC labeled antibodies of goat specific for rabbit globulin showed a general fluorescence that was more intense on young hyphae and conidia. Strong apple green fluorescence of young hyphae and sclerotia of *Corticium invisum* (Chakraborty and Das Biswas, 2008), *Macrophomina phaseolina* (Chakraborty *et.al.* 2012) and *Sclerotium rolfsii* (Bhagat and Chakraborty, 2020) with homologous PAb were evident in indirect immunofluorescence test. Similarly, treatment of mycelia and spores of *Exobasidium vexans* (Chakraborty and Sharma, 2007), *Glomerella cingulata* (Chakraborty *et.al.* 2008a) with their respective homologous PAbs followed by FITC labeling developed a strong fluorescence that was more intense on young hyphal tips and spore wall. In case of *Pestalotiopsis disseminata* spores, only the setulae and appendages showed apple green fluorescence as the conidia are dark septate, confirming the identity of the pathogen (Acharya *et al*. 2015). Immunodetection of *Aspergillus flavus* (Chakraborty *et al.* 2008b) and *Aspergillus niger* (De Palit *et al.* 2023), dominant isolates of stored seeds of *Vigna radiata*, *Cajanus cajan* and *Lens culinaris* have been reported. Different test formats including DIBA, PTA-ELISA and indirect immunofluorescence have been used for early detection of fungal pathogens of different crops for developing disease management strategies using bioinoculants (Chakraborty and Chakraborty, 2021). Immunodetection of *Rhizophagus fasciculatus* and *Gigaspora gigantea* in soil and root tissues in *Citrus reticulata*, their exploitation as bioinoculants and cellular localization of defense enzymes following induced immunity developed against *Fusarium solani* have also been documented (Chakraborty and Allay, 2022).

DECLARATIONS

Conflict of interest: Author declares no conflict of interest.

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