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# Pathogenicity and endophytic activity of white muscadine fungus, *Beauveria* bassiana (Balsamo) Vuillemin against Fall armyworm, Spodoptera frugiperda (J. E. Smith) in maize, Zea mays L.

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ABSTRACT: Fall armyworm, *Spodoptera frugiperda* (J.E. Smith) has gained the major pest status in maize throughout the country. Management of Fall armyworm became difficult because of its cryptic habitat inside maize whorls and polyphagous nature. Entomopathogenic fungus (EPF), *Beauveria bassiana* has immense potential as bioagents owing to their broad host range, and pathogenicity under laboratory conditions. Present study involves the exploitation of EPF for effective management of fall armyworm which includes identification of virulent strain of EPF, testing the pathogenicity under lab conditions. The isolates collected from different locations during survey are identified morphologically as *Beauveria bassiana* and were characterized by PCR based DNA sequencing analysis. The result showed that the five isolates showed slight variations in their growth pattern but no differences were observed at morphologically level. PCR based DNA sequencing proved that all isolates, irrespective of insect hosts and locations were found to be identified as *B. bassiana* at molecular level. The *B. bassiana* isolates were tested for pathogenicity and endophytic activity against Fall armyworm under laboratory conditions. The results showed that Bb TM isolate was capable of causing highest mortality of 66.67, 60.00 and 53.33 per cent against first, second and third instar with LC<sub>50</sub> values of 2.51 × 10<sup>5</sup>, 2.05 × 10<sup>6</sup> and 4.56 × 10<sup>7</sup> respectively. None of these *B. bassiana* isolates proved to be endophytic in maize plants.

Keywords: Beauveria bassiana, endophyte, Spodoptera frugiperda, bioassay, LC<sub>50.</sub>

# INTRODUCTION

Maize (Zea mays L.) is the third most important cereal crop in India after rice and wheat which accounts for around 10 per cent of total food grain production in the country. The Fall Armyworm (FAW), Spodoptera frugiperda (J. E. Smith) of the family Noctuidae, Lepidoptera is a serious yield constraint in this crop (Kalleshwaraswamy et al., 2018). The fall army worm feeds on all the growth stages of maize starting from seedling emergence to cob development (Sisay et al., 2019). Managing fall armyworm has been a great challenge due to their high fecundity and large migratory range (Westbrook et al., 2016). Efforts to manage through indiscriminate use of insecticides may result in development of resistance besides being harmful to environment and natural enemies. Entomopathogens proved as an effective strategy in many situations of severe pest outbreak and reports on the management of FAW using entomopathogens have been reported (Lezama-Gutierrez et al., 2001 and Guo et al., 2020). The white muscardine fungus, Beauveria bassiana (Balsamo) Vuillemin is the most studied entomopathogenic fungi widely applied against agricultural insect pests due to broader host range (Ownley et al., 2004; McGuire and Northfield, 2020). In the present investigation, the EPF were exploited during survey and their pathogenicity and endophytic activity were evaluated against *S. frugiperda* in maize under controlled laboratory conditions.

# MATERIALS AND METHODS

**Survey for collection of entomopathogenic fungi.** Extensive survey was carried out in different regions of Tamil Nadu to collect and isolate the naturally occurring entomopathogens on insects from January 2020 to December 2021. The details of survey location and collected insect cadavers are described in Table 1.

Isolation of fungal isolates. For isolation of fungi, mycosed samples were surface sterilized using 70 per cent ethanol (30 seconds) followed by 1.5 per cent sodium hypochlorite (60 seconds) and then thoroughly washed with sterilized distilled water several times (Goettel and Inglis, 1997). The excess water was removed by keeping the cadaver in sterilized Whatman filter paper No. 1. The cadavers were then cut into small pieces with the help of sterile blade and the bits were aseptically transferred with sterilized inoculation needle on to 90 mm sterilized petri plate containing Potato Dextrose Agar (PDA) with streptomycin sulphate (0.5g/ l) and incubated at  $25\pm2^{\circ}$ C. The stock cultures were sub-cultured on PDA medium at 15 days interval and the virulence was revived by passing through the host after every 5 to 6 times of subculturing.

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District	Place of visit	GPS Location	Crop	Stage of the crop	Host	Cadavers collected
Enada	Arachalur	11.1525° N and 77.4358° E	Maize	Tasseling stage	-	-
Erode	Modakurichi	11.2382° N and 77.7702° E	Maize	Cob formation	-	-
Dharmapuri	Palaya papparapatti	12.1312° N and 78.340° E	Maize	Cob formation	-	Beauveria bassiana
	Morappur	12.838° N and 78.2257° E	Maize	Vegetative stage	-	-
Krishnagiri	Ettipatti	12.206° N and 78.2955° E	Maize	Cob formation	-	-
Trichy	Pullambadi	10.5635° N and 78.553° E	Maize	Vegetative stage	-	-
Karur	Edaiyapatty	10.5715° N and 78.2269° E	Maize	Tasseling stage	-	-
Thiruyannamalai	Perumanam	12.0315° N and 79.1028° E	Maize	Tasseling stage	-	-
Tintuvaimainaiai	Vazhavachanur	12.0741° N and 78.9845° E	Maize	Cob formation	-	-
Thoniounr	Mapillainaicken patty	10.4232° N and 78.522° E	Maize	Vegetative stage	-	-
manjavui	Thirukanurpatti	$10.4313^\circ$ N and 79.51° E	Maize	Vegetative stage	-	-
Animalum	Ottakovil	11.1256° N and 79.60° E	Maize	Vegetative stage	-	-
Ariyalur	Sendurai	11.1532° N and 79.1014° E	Maize	Vegetative stage	-	-
Perambalur	Suriyur	10.3620° N and 78.3738° E	Maize	Vegetative stage	-	-
	Pasumbalur	11.4361° N and 78.9080° E	Maize	Cob formation	-	-
Pudukottai	Gandharvakottai	10.3334° N and 79.329° E	Maize	Vegetative stage	-	-
Villupuram	Chinna Salem	11.3732° N and 78.5118° E	Maize	Vegetative stage	-	-
	Periyanesalur	11.3230° N and 79.519° E	Maize	Cob formation	-	-
Cuddalore	Avatti	11.2835° N and 79.359° E	Maize	Vegetative stage	-	-
Therei	Periyakulam	10.32° N and 77.309° E	Maize	Vegetative stage	-	-
Them	Uthamapalayam	$9.9531^\circ$ N and $77.4074^\circ$ E	Maize	Vegetative stage	-	-
	Othakkadai	9.5814° N and 78.1224° E	Maize	Tasseling stage	-	-
Madurai	Kondayampatti	10.0538° N and 78.0303° E	Maize	Vegetative stage	-	-
	Thondamuthur	11.0058° N and 76.8235° E	Cabbage	Vegetative stage	Plutella xylostella	B. bassiana
Coimbatore	Madampatti	10.9698°N and 76.8598°E	Bhendi	Vegetative stage	Unknown beetle	B. bassiana
	TNAU	TNAU 11.0122°N and 76.9354°E	Mulberry	Vegetative stage	Bombyx mori	B. bassiana
			Cauliflower	Curd formation	Hellula undalis	B. bassiana
Tuticorin	Killikulam	$8.7063^\circ$ N and $77.8614^\circ$ E	Maize	Vegetative stage	-	-
	Kovilpatti	9.1504° N and 77.8529° E	Maize	Cob formation	-	-
Tirupelveli	Vellappaneri	9.0462° N and 77.7057° E	Maize	Tasseling stage	-	-
Thunciven	Kanarpatti	8.8827° N and 77.6553° E	Maize	Cob formation	-	-
Tenkasi	Pattakurichi	9.2088° N and 77.4528° E	Maize	Cob formation	-	-
Tirupur	Kethanor	10.8757° N and 77.2732° E	Maize	Silk formation	-	-
Ramanathapuram	Karisalkulam	9.4188° N and 78.2601° E	Maize	Cob formation	-	-

Table 1: Entomopathogenic fungi collected and identified during the survey (2019-2022).

**Morphological identification of fungal isolates.** Fungal pathogens grown on PDA plates for about 15 days were used for the morphological identification. Phase contrast microscopy was used to examine morphological characteristics such as colony colour, mycelial character, spore size and structure. The growth parameters like radial growth, spore count and days taken to cover up the full plate were also observed.

**Molecular characterization.** Morphological identified fungus was further confirmed by molecular studies. For molecular characterization, CTAB method described by Dhar *et al.* (2019) was followed. The fungal isolates were grown in 100 ml Potato dextrose broth and incubated for seven days. The mycelial mat was then removed carefully and wash three times with sterile

distilled water to remove media constituents. Fresh mycelial mass (100 mg) of each isolate was ground separately in liquid nitrogen using pestle and mortar, followed by addition of 5 to 6 ml cetyl trimethyl ammonium bromide (CTAB) extraction buffer (10 mM tris-base (pH 8.0), 20 mM ethylene diamine tetra acetic acid (EDTA) (pH 8.0), 1.4 M NaCl, CTAB (2%), mercapto ethanol (0.1%)) and were incubated in dry water bath at 65°C for 30 minutes. The solution was extracted with 5 ml of chloroform: iso amyl alcohol (24:1) and centrifuged at 10000 rpm for 10 minutes. After centrifugation, the supernatant was mixed with equal volume of ice-cold isopropanol for DNA precipitation. The precipitated DNA was washed with 70 per cent ethanol by centrifuging the contents at

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12000 rpm for 5 minutes. Further, the obtained DNA pellets were air dried for 30 minutes and suspended in 100  $\mu$ l TE (10 mM Tris, 1mM EDTA, pH 8.0) buffer. The DNA concentration was estimated using a Nano drop Spectrophotometer (Thermo Scientific Company, USA) as per the procedure by Desjardins and Conklin (2010).

PCR amplification of ITS1-5.8S-ITS2 and 18S genes of fungal isolates was performed with universal primers (Wu 2001). ITS1 et al., (5'-TCCGTAGGTGAACCTGCGG-3') as forward primer, (5'-TCCTCCGCTTATTGATATGC-3') ITS4 as reverse primer were used. PCR amplifications were performed on gradient Master cycler (Eppendorf, Germany) with a total volume of 50 µl which included 5 µl 10X Taq-DNA polymerase reaction buffer, 1 µl (1 mM) dNTPs, 1 µl (20 pmol) each of the opposing amplification primers, 0.5 µl (5 u/ µl) Taq-DNA polymerase, and 50 ng genomic DNA and sterile distilled water. The PCR program involved 40 cycles of initial denaturation at 94°C for 3 min; followed by 35 cycles of 94°C for 1 min, 56°C for 30 sec, 72°C for 1 min and final extension at 72°C for 10 min. Successful PCR amplifications were determined by 1.5 per cent agarose gel, viewed under UV light and documented in an Alpha Imager. The PCR products were then Sanger sequenced by Eurofins Genomics (Bangalore, India). The nucleotide sequence obtained was aligned for its similarity using the BLAST software

**Phylogenetic analysis.** The native isolates obtained in the present investigation was analysed with 15 other isolates of *B. bassiana* from GenBank database. An isolate of *Metarhizium anisopliae* were considered as outgroup for better phylogenetic reconstructions. The indigenous isolates were sequenced for the partial region of internal transcribed sequence and the rest of the sequences were obtained from GenBank database entries (http://www.ncbi.nlm.nih.gov/Genbank/ index.html). The sequences were initially aligned using the multiple alignment program MEGA ver. 7 and the flanking regions that were not a part of significant multiple alignment were trimmed off. The statistical procedure of neighbour joining (NJ) of the program MEGA ver. 7 was used and distances for neighbour joining tree were calculated under the Kimura 2parameter model.

Pathogenicity test. Five isolates collected during the survey and two isolates from Insect pathology lab, Department of Agricultural Entomology, TNAU were evaluated for their pathogenicity against Fall Armyworm. Fungal spore suspension of the seven isolates were prepared by scrapping spores from sporulated plates and were suspended in 10 mL sterile distilled water containing 0.05 per cent Tween 80<sup>®</sup>. The spore count in the liquid suspension was assessed using improved Neubauer haemocytometer. Larvae were treated with different concentration of B. bassiana isolates containing 0.05 per cent Tween 80<sup>®</sup> using hand atomizer (Sánchez-Peña et al., 2007). Larvae treated with 0.05 per cent Tween 80<sup>®</sup> served as untreated control. Treated larvae were individually allowed to feed on artificial diet in plastic containers. The experiment was performed in completely randomized design with seven treatments replicated three times. Thirty number of different instars (first, second and third) were released for each treatment and incubated at room temperature of 25±2°C. Percentage mortality was corrected (Abbott, 1925) and statistically analyzed after necessary transformation. The list of isolates used in the study are presented in Table 2.

Sr. No.	Isolates	Substrate / host	Location	Accession number
1.	Bb1	Plutella xyllostella	Coimbatore	MZ853723
2.	Bb2	Hellula undalis	Coimbatore	MZ749648
3.	Bb3	Spodoptera frugiperda	Dharmapuri	MZ749646
4.	Bb4	Bombyx mori	Coimbatore	ON202897
5.	Bb5	Unknown beetle	Coimbatore	ON202916
6.	Bb TM	Helicoverpa armigera	Dharmapuri	MH590235
7.	Bb BR	Leucinodes arbonalis	Coimbatore	MK918495

Table 2: Details of *B. bassiana* isolates used in the present study.

Endophytic study using seed treatment method. The endophytic activity of fungus on maize was done by following the slight modification in procedure as described by Ramos et al. (2020); Russo et al. (2021). The maize seeds were surface sterilized with 2 per cent sodium hypochlorite (one minute) followed by rinsing in 70 per cent ethanol (1 minute) and several washes with sterile distilled water. The surface-sterilized seeds were soaked in 10 ml fungal suspension of each isolate for 24 hours. Seeds were then dried on sterile filter paper under aseptic conditions for 30 minutes before being placing them in sterile germination paper using roll paper towel method. For control, the seeds were soaked in sterile water with 0.01% Tween 80. When the maize plants reached two leaves unfolded stage, different parts of maize plants including roots, stems, and leaves were collected and surface sterilized with 1.5 per cent sodium hypochlorite (3 minutes), 70 per

cent ethanol (2 minutes) and sterile distilled water for three times (one minute). The effectiveness of sterilisation was tested by inoculating PDA medium with 100  $\mu$ l of the last rinse water.

To avoid the death of endophyte tissue, damaged plants from the sterilising procedure were eliminated. Plants were then dried in sterile paper towels and chopped into small (1 cm2) pieces aseptically in a laminar flow hood. Afterwards, the plant pieces were inoculated on PDA culture media in Petri plates (9 cm diameter) with streptomycin sulphate (250 mg/l) and incubated for 3 days. To obtain colonies free of contamination, a single spore from each EPF was harvested and re-inoculated on PDA growth medium.

### RESULTS AND DISCUSSION

In the present investigation, B. bassiana was isolated from Fall armyworm from maize fields in Tamil Nadu. Another study conducted by Ramanujam et al. (2021) in Karnataka confirmed the natural occurrence of Beauveria species in Spodoptera frugiperda in the maize fields which is similar to the findings of the current investigation. The fungal pathogens isolated from various insect cadavers were observed under phase contrast microscope for studying the morphological characters of the colonies. The observations made on the colony characters include growth pattern, colony colour, surface texture, mycelial structure, conidia shape and size (Table 3).

	Morphological characters						
Isolate code	Growth pattern	Colony colour	Reverse colony colour	Surface texture	Mycelial structure	Conidia shape	Conidia size(µm)
Bb1	Disperse, dense	Yellowish white	white	Smooth, powdery	Hyaline, septate	Globose	2.03
Bb2	Disperse, dense	white	Yellowish white	Smooth, powdery	Hyaline, septate	Globose	2.33
Bb3	Disperse, dense	Yellowish white	white	Smooth, powdery	Hyaline, septate	Globose	2.22
Bb4	Disperse	white	white	Smooth, cottony	Hyaline, septate	Globose	2.46
Bb5	Raised, disperse	white	white	Smooth, powdery	Hyaline, septate	Globose	1.99
Bb TM	Flat and dense	white	white	Smooth, powdery	Hyaline, septate	Globose	2.11
Bb BR	Flat and disperse	white	Yellowish white	Smooth, powdery	Hyaline, septate	Globose	2.05

Table 3: Morphological characterization of Beauveria bassiana isolates.

The results revealed that all the five isolates have hyaline and septate mycelium with whitish or yellowish white colony which are the characters of the B. bassiana. The isolates viz., Bb1, Bb2, Bb3. Bb5, Bb TM and Bb BR had smooth and powdery surface whereas, the isolate Bb4 showed smooth cottony appearance. The surface texture of isolates is smooth, powdery or cottony with globose conidia and their size ranges from 1.99-2.46 µm. B. bassiana isolates were morphologically confirmed by the presence of white or yellowish white colony, globose or sub-globose conidia (Talaei-Hassanloui et al., 2006 and Fernandes et al., 2006). These characters confirmed that these indigenous isolates used in the present study were B. bassiana. In addition, as shown in Table 4, observations were also taken on growth factors such as radial growth, sporulation, and the number of days to cover the petri plate. After 15 days of incubation, the radial

growth of B. bassiana isolates ranged from 5.53 to 8.61 cm, with the isolates Bb TM (8.61 cm) and Bb3 (8.56 cm) showing significantly greater growth rates. Bb1 had the highest spore count of  $12.34 \times 10^8$  spores mL<sup>-1</sup>, followed by Bb3, which had the spore count of  $11.68 \times$  $10^8$  spores mL<sup>-1</sup>. In isolates, Bb4 ( $5.53 \times 10^8$  spores mL<sup>-1</sup> <sup>1</sup>) and Bb5 (5.85  $\times$  10<sup>8</sup> spores mL<sup>-1</sup>), the lowest spore count was detected. The time took to cover the entire petri plate ranged from 14 to 17 days. The present study also conclude that isolate Bb TM had greater growth and spore production ability and was efficient in controlling Fall armyworm under laboratory conditions. Similarly, the studies made by Zhang et al. (2011) have shown that morphological characteristics of B. bassiana such as growth rate, colony color, texture and spore production are correlated with virulence against target pest.

Table 4: Growth characters of Beauveria bassiana isolates.

Isolate name	Mean growth (Diameter in cm)	Sporulation (1×10 <sup>8</sup> spores/mL)	Days taken to cover full petri plate
Bb1	7.42 (2.72) <sup>b</sup>	12.34 (3.51) <sup>a</sup>	14
Bb2	6.30 (2.51) <sup>c</sup>	10.87 (3.30) <sup>d</sup>	15
Bb3	8.56 (2.89) <sup>a</sup>	11.68 (3.42) <sup>b</sup>	14
Bb4	5.53 (2.35) <sup>d</sup>	8.10 (2.85) <sup>e</sup>	14
Bb5	5.85 (2.40) <sup>cd</sup>	6.14 (2.48) <sup>f</sup>	17
Bb TM	$\frac{8.61}{(2.94)^{a}}$	11.17 (3.34) <sup>c</sup>	14
Bb BR	$\frac{8.32}{(2.90)^a}$	10.84 (3.29) <sup>d</sup>	15

Figures in parentheses are square root transformed values. In a column, means followed by the common letter(s) are not significant by LSD (p <0.05).

Morphological characters are mostly used for identification at genus level but species level identification is necessary for further advanced study. Kiruthiga et al., Biological Forum – An International Journal 14(2): 967-973(2022)

The 18S rRNA genes of isolates were amplified using universal primers viz., ITS1 and ITS4. The nucleotide sequences of the five isolates were deposited in the Gen 970

Bank and allotted with the accession numbers (Table 2). The homology search against the GenBank data base revealed 96.92 to 99.60 per cent similarity to the ITS region of *B. bassiana* as in Table 5. The isolates were thus designated as *B. bassiana* with high similarity and could not be distinguished. Dhar *et al.*, (2019) observed the amplification of ITS region of *B. bassiana* isolates at 524 bp which is in line with the findings of the current study as the amplification of ITS region was around 550 bp. The phylogenetic tree was

obtained by the sequence analysis of ITS region, of all the study isolates and the sequences of 5 other species obtained *Beauveria* from NCBI, GenBank. Phylogenetic tree based on the ITS sequences clustered into two groups. The study isolates Bb4 (ON202897) and Bb5 (ON202916) were grouped together in a cluster. *Beauveria bassiana* isolates Bb2 (MZ749648), Bb1 (MZ853723) and Bb3 (MZ749646) were grouped as three separate clusters along with other reference sequences retrieved from GenBank as in Fig. 1.



Fig. 1. Phylogram obtained with NJ analysis of internal transcribed spacer (ITS) sequences from 19 isolates of Beauveria bassiana. Metarhizium anisopliae was used as outgroup.

Among the seven isolates tested, the results revealed that Bb TM isolate (MH590235) was capable of causing higher mortality against S. frugiperda under laboratory conditions. Larval mortality was dose dependant and percent mortality increased with concentration and time of treatment. At nine days after treatment, mortality ranged from 20.00 to 66.67 per cent, 13.33 to 60.00 per cent, 10.00 to 53.33 per cent for first, second and third instar respectively as in Fig. 2. Highest mortality of 66.67 per cent was recorded for first instar at 10<sup>8</sup> conidia/ ml after nine days after treatment. Concentration mortality responses of first, second and third instar to Bb TM isolate (MH590235) showed that LC<sub>50</sub> values of  $2.51 \times 10^5$ ,  $2.05 \times 10^6$ , 4.56  $\times 10^7$  conidia/ml with a fiducial limit of  $10^3 - 10^7$ ,  $10^4 - 10^7$  $10^7$  and  $10^6 - 10^8$  conidia/ml. Carneiro *et al.* (2008) isolated 24 isolates of Beauveria spp from mycosed cadavers in maize ecosystem and found that B. bassiana isolates were capable of causing 63.0 to 100 per cent mortality in Fall Armyworm which is in accordance with the present study as the isolate MH590235 (Bb TM) caused a maximum mortality of 66.67 per cent in laboratory conditions. Also, Rajula et al. (2021) proved that six isolates of B. bassiana were effective in controlling Fall armyworm under laboratory conditions with mortality ranging from 55 to 91.67 per cent. None of the *B. bassiana* isolates showed endophytic activity in maize plants in present study. In contrast to present findings, Ramirez-Rodriguez and Sanchez-Pena (2016) recovered endophytic *B. bassiana* from 65.3 per cent of fungus treated maize seeds.



Fig. 2. Dose-mortality responses of FAW treated with Bb TM isolate (MH590235).

Table 5: Identification of	Beauveria bassiana	by partial	sequencing of 1	8S rRNA genes.
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Sr. No.	Isolate name	Matching organism in NCBI genebank database with accession number	Percent similarity code	E Value
1.	Bb1	B. bassiana (MT635019)	99.60	0.0
2.	Bb2	B. bassiana (MZ824408)	99.24	0.0
3.	Bb3	B. bassiana (MZ749645)	96.92	0.0
4.	Bb4	B. bassiana (MZ824399)	99.45	0.0
5.	Bb5	B. bassiana (MK049987)	98.20	0.0

### CONCLUSIONS

The current study identified *B. bassiana* isolate for the management of Fall armyworm, *S. frugiperda*. Future studies should be made on cost-effective mass production, formulation, shelf-life studies and safety of fungus to nontarget organisms under laboratory and field conditions for the successful biological control of Fall armyworm.

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