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Evaluation of a native isolate of *Metarhizium anisopliae* (Metschn.) Sorokin TMBMA1 against tea mosquito bug, *Helopeltis theivora* infesting cocoa (*Theobroma cacao* L.)

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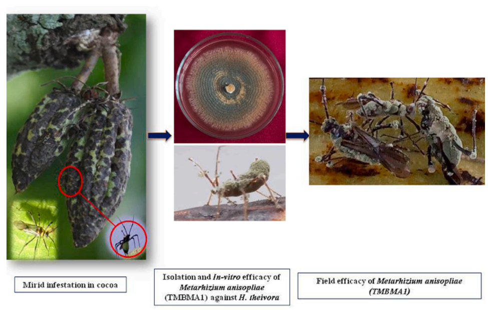
HIGHLIGHTS

- Native strain of *Metarhizium anisopliae* isolated from naturally infected adult of *Helopeltis theivora*.
- In cage and field trials, *M. anisopliae* reduced *H. theivora* incidence in cocoa.
- A *M. anisopliae* proved more effective in field condition by protecting the cocoa pods against *H. theivora* infestation.

GRAPHICAL ABSTRACT



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ABSTRACT

The tea mosquito bug, *Helopeltis* spp. is a destructive pest of cocoa across the globe. In the climate change scenario, the tea mosquito bug species complex, *viz. Helopeltis theivora* Waterhouse, 1886, *H. bradyi* Waterhouse, 1886, and *H. antonii* V. Signoret, 1858, poses a serious threat to cocoa cultivation in India. Presently, systemic and/or broad-spectrum insecticides are used for mirid management by cocoa growers. However, concerns about their adverse effects on human health and the environment prompted us to search for alternative eco-friendly options for their management. Entomopathogenic fungi (EPF) are an effective and eco-friendly alternative to chemical insecticides for the management of cocoa mirids. The use of native strains of biological control agents improves their success rate. Hence, the present investigation was conducted to isolate, characterize and evaluate an indigenous insect-pathogenic isolate of EPF *Metarhizium anisopliae* against the mirid *H. theivora*. An extensive survey was conducted in the Dakshina Kannada district of Karnataka, India, to collect the adults of *H. theivora*

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naturally infected with entomopathogenic fungi. A pure culture of the EPF named as TMBMA1 was isolated from the infected adults. Based on the detailed morphological and molecular characterization, the isolate TMBMA1 was identified as *Metarhizium anisopliae* (Metsch.) Sorokin. The isolate TMBMA1 was found to be highly effective against *H. theivora* in an *in-vitro* bioassay and caused 100% insect mortality at 1×10^8 and 1×10^9 conidial suspensions 6 days post inoculation. Similarly, TMBMA1 was found effective in significantly reducing the incidence of mirids in field evaluations conducted for two consecutive years. TMBMA1 was found better than other commercial biopesticides and was comparable with the recommended chemical insecticide Lambda-cyhalothrin. In conclusion, our results reveal that the native EPF, *M. anisopliae* isolate TMBMA1 has great potential against the mirid *H. theivora* and can serve as an eco-friendly and sustainable alternative for mirid management in cocoa and other plantations in India.

1. Introduction

Cocoa (*Theobroma cacao* L.), a native of South and Central America, is an important cash crop cultivated in several humid tropical countries around the globe. It was first introduced into India in 1798 (Ratnam, 1961). As an understorey crop, cocoa is commercially cultivated as an intercrop in arecanut, coconut, and oil palm plantations of the southern states viz., Karnataka, Kerala, Tamil Nadu, and Andhra Pradesh. Currently, cocoa is cultivated in an area of 97,563 ha in India, with an annual production of 25,783 tonnes (DCCD, 2020). However, West Africa leads global production (more than 70% of the total world production), followed by South-East Asia and Latin America (Lahive et al., 2019). Cocoa is a key ingredient for the chocolate industry and other confectionaries. The demand in India for dry cocoa beans is about 30,000 tonnes per annum. However, the domestic production of cocoa falls short of meeting the projected demand. Hence, India is importing a major share of its requirements from other cocoa-growing countries (DCCD, 2020).

Besides this, cocoa cultivation is interlaced with serious threats due to several biotic and abiotic factors. Among them, insect pests and diseases are the key factors limiting the production and productivity of cocoa. Among the biotic stresses, the tea mosquito bug (mirid), *Helopeltis* spp. (a species complex; Heteroptera: Miridae), has emerged as a major pest of cocoa in South India (Thube et al., 2019). In this species complex, *H. theivora* dominates cocoa plantations in India with more than 40% yield reduction potential. This mirid infests the economically important part of the cocoa tree, *i.e.,* the pod, and even insignificant feeding damage caused by the insect pest may lead to heavy quantitative as well as qualitative losses (Muhamad and Way, 1995).

In order to safeguard the cocoa crop from mirid infestation, cocoa growers resort to periodical, indiscriminate use of insecticides. Besides affecting human health, these chemical insecticides are also responsible for environmental pollution in the fragile plantation ecosystem incurring enormous setbacks to natural resources. A total of nine pesticide residues (Lindane, Dieldrin, Aldrin, Gamma-hexachlorocyclohexane, Dichlorodiphenyldichloroethylene, Dichlorodiphenyldichloroethane, DDT, Beta HCH, and Heptachlor) were detected in dry cocoa bean samples collected from different mega stores of Nigeria (Oluwatuyi et al., 2020). The organochlorine pesticide residues (aldrin, dieldrin, lindane, beta-HCH, p,p'-DDE, p,p'-DDD, p,p'-DDT, and methoxychlor) were detected in fermented cocoa beans collected from Dormaa West District of Ghana (Okoffo et al., 2016). At present, the hindrance of ecosystem health by agrochemicals is an emerging concern countering the one-health concept. Of late, residues from neonicotinoid insecticides (Imidacloprid and Thiamethoxam) were detected in soil, water, and people's hair in the Philippines (Bonmatin et al., 2020). Moreover, the occurrence of pesticide residue in dried cocoa beans is hampering international trade and global agri-business. All these consequences warrant a safer non-chemical alternative for the management of the mirid species complex in cocoa. Biological control offers all these benefits and aims at safer crop protection. Among the biological control agents (BCAs), entomopathogenic fungi have been explored extensively due to their potential biocontrol ability, self-perpetuating behaviour, eco-friendly nature, easy mass multiplication, and species-specific action. Entomopathogenic fungi cause lethal infections in their host and act as a natural regulator of many pests, including soil-inhabiting insects by epizootics (Goettel et al., 2005). These microorganisms have attracted significant attention for their use in biological control programs of insect pests in a diverse ecosystem (Lacey et al., 2015; Strasser et al., 2010). Among the EPF, *Metarhizium anisopliae* is the most studied fungus, along with *Beauveria bassiana* (Balsamo) Vuillemin, in terms of commercial production (Goettel et al., 2005; Meyling and Eilenberg, 2007). Several microbial pesticides have been formulated from different species of EPFs worldwide (Fang et al., 2014). Among these, the genus *Metarhizium* comprises generalist and specialist strains based on their host range (Moonjely and Bidochka, 2019). *Metarhizium anisopliae*, *M. brunneum*, and *M. robertsii* fall under generalists and infect insect species from several orders, whereas *M. acridum* is a specialist and infects insect species specific to order Orthoptera (Wang et al., 2016). However, both groups have a similar mode of action *viz.*, spore adhesion, spore germination, cuticle penetration, mycelial dissemination through body hemolymph, outgrow over the cadavers of dead insects, and production of disseminative green conidial masses for horizontal dispersion to other insects (San Aw and Hue, 2017). Apart from managing lepidopteran insect pests, *M. anisopliae* was found effective in the management of many sucking pests *viz*., leafhopper (Naik and Sekharappa, 2009), *Thrips tabaci* (Gillespie, 1986) and *Frankliniella occidentalis* in chrysanthemum (Lopes et al. 2000). This cosmopolitan species infests insect hosts ranging from termites and cockroaches to pests including whiteflies and soil-dwelling root grubs (Maniania and Ekesi, 2013; Zimmermann, 2007). *Metarhizium anisopliae* was also proved effective to manage the population of red spider mite *Oligonychus coffeae* and wood eating termite *Microcerotermes beesoni* infesting tea plantations of India (Kumhar et al. 2020). Notwithstanding this wide host range, it is always advised to use the native strain of any BCA to achieve maximum success. Thus, identification of locally isolated strains through pathogenicity screening has paramount importance in increasing the chance of obtaining successful microbial biopesticides. Hence, the present study aimed to isolate, characterize and evaluate an indigenous insect-pathogenic isolate of *M. anisopliae* collected from field infected adults of the mirid, *H. theivora*.

2. Material and methods

2.1. Survey and sample collection

An extensive field survey was conducted in ten different cocoa-growing villages of Dakshina Kannada district of Karnataka, India, during the year 2018 (Fig. 1). The dead cadavers of tea mosquito bug (TMB), *H. theivora* adults infected with fungi were collected. Specimens were placed in sterile insect collection tubes, labeled properly, and transferred to the laboratory for further investigation.

2.2. Single spore isolation

The naturally dead cadaver of *H. theivora* was surface sterilized using 2% sodium hypochlorite solution for 10 s and washed thrice with sterile distilled water to remove the traces of sodium hypochlorite. Surface sterilized insect cadavers were placed onto potato dextrose agar (PDA) medium and incubated at 28 ± 2 °C for 5 days. Sub-culturing of single colonies was executed to obtain pure cultures of the fungus. These pure colonies were maintained on the agar slants at 4 °C for further use.

2.3. *Morphological characterization*

Morphological and cultural characteristics of entomopathogenic fungi were recorded according to Fernandes et al. (2010). The two-week-old culture was inoculated in the centres of fifteen Petri dishes containing PDA medium and incubated at 28 ± 2 °C in the dark. The macroscopic characteristics of the colonies, such as colony pattern, colour, and growth rate, were recorded 14 days post inoculation. Morphometric and morphological characteristics like measurements and shape of conidia and arrangement of spores on conidiophores were recorded using the wet mount glass slide microscopic technique, using a trinocular research microscope with an image analyser (Nikon Eclipse Ni-U, Tokyo, Japan). Simultaneously, an active culture was submitted to the Agharkar Research Institute, Pune, for proper identification based on morphological taxonomy. The pure culture of the fungus designated as TMBMA1 (*Metarhizium anisopliae* isolate 1 identified from TMB) is deposited in the fungal culture collection of the Entomology lab at ICAR-Central Plantation Crops Research Institute, Regional Station, Vittal, Karnataka.

2.4. ***Molecular** characterization*

Morphological identification of EPF isolate TMBMA1 was further confirmed using the amplification of ITS-rDNA region. Total fungal DNA was extracted from the mycelial mat using the CTAB method with minor modifications (Pandian et al., 2018). PCR reactions were performed to amplify the partial rDNA gene sequences with a 50.0 μl reaction volume containing 25.0 μl of Master Mix (Promega Corporation, USA), 2.0 μl each of forward ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990) primers (10 pmol/μl), 2.0 μl of DNA, and 19.0 μl of sterile distilled water. The amplification profile was carried out using a Bio-Rad T100 thermocycler, which was preheated at 95° C for 3 min, followed by 35 cycles of 95° C for 30 sec, 55° C for 30 sec, 72° C for 1 min, and final extension at 72° C for 5 min. The amplified product was visualized using a 1% agarose gel (Sambrook and Russell, 2001), purified using the Qiagen Gel Purification Kit, and the DNA fragment was sequenced with Sanger's sequencing (AgriGenome Labs Pvt. Ltd., Kochi, India). The obtained sequences were aligned using BioEdit (Biological sequence alignment editor, https://www.mbio.ncsu.edu/BioEdit/bioedit.html, RRID: SCR_007361) (Hall, 1999) and compared with the available sequences in NCBI (https://www.ncbi.nlm.nih.gov/BLAST/). The sequence was deposited in the NCBI GeneBank. The phylogenetic relationship was established among the related species of *Metarhizium* using *Cordyceps japonica* (DQ522547) as an outgroup. Details of the nucleotide sequences used for phylogenetic analysis are given in supplementary Table 1. Phylogenetic analysis was performed with MEGA10 software (Kumar et al., 2018) using the Maximum likelihood (ML) method with a bootstrap of 1000 replicates.

2.5. ***EPF mass**-culturing*

A mycelial disc of about 5 mm of pure EPF culture was placed into 50 ml of potato dextrose broth (PDB) and incubated at 28 ± 2 °C for 5–7 days under shaking conditions (150 rpm). The spore suspension was harvested through two layers of muslin cloth to remove the remnants of media. The resulting spore suspensions were adjusted to the concentrations of 1×10^3 to 1×10^9 conidia ml^{-1} using Neuberger hemocytometer under Leica DM LB2 compound microscope (Leica

Village	Latitude	Longitude
Murulya	12° 41' 17.717"	75° 24' 52.362"
Boonadka	12° 41' 57.541"	75° 36' 3.480"
Alankar	12° 46' 42.289"	75° 20' 54.181"
Jogogara	13° 32' 41.437"	75° 23' 45.535"
Khandya	13° 32' 52.566"	75° 25' 7.636"
Enekallu	12°40' 45.29"	75° 29' 32.913"
Periyadka	12°49' 18.905"	75° 17' 41.812"
Bantra	12°44' 31.884"	75° 30' 1.681"
Puttur	12°48' 31.849"	75° 16' 1.18"
Vittal	12° 46' 43.60"	75° 06' 58.60"

Fig. 1. Schematic depiction of cocoa plantations surveyed in Dakshina Kannada district of Karnataka, India.

Microsystems Wetzlar GmbH, Germany).

2.6. *Insect rearing*

Aluminium insect rearing cages were fabricated (Thube et al., 2019) for the maintenance of *H. theivora* population under laboratory conditions. Gravid mirid females were collected from the cocoa fields, and species-level identity was confirmed using the keys as described by Stonedahl (1991). Gravid females were allowed to lay their eggs on fresh cocoa pods kept in the rearing cages. The respiratory processes of eggs projecting from the surface of the pods were indicative of the presence of eggs. Newly hatched nymphs were transferred into nymphal rearing cages containing fresh pods. Freshly emerged adults were collected for evaluating *M. anisopliae* at various concentrations.

2.7. *Evaluation of entomopathogenic activity*

2.7.1. *Laboratory bioassays*

The efficacy of EPF isolate TMBMA1 was evaluated against adults of *H. theivora* in an *in-vitro* bioassay using seven different conidial concentrations ranging from 1×10^3 to 1×10^9 conidia ml^{-1}. Ten freshly emerged adults were transferred to a 90 mm Petri dish using a camel hairbrush. Then, 5 ml of a desired conidial suspension was poured into the Petri dish. The Petri dish was shaken for approximately 15 s for maximum exposure of adults to the conidial suspension. These inoculated adults (10 numbers) were treated as one replication. Four such replications were maintained for each concentration of conidial suspension (total insects, n = 40 per treatment). Inoculated adults were transferred to separate aluminium rearing cages containing uniform aged (5 months) surface sterilised cocoa pods. The experiment was repeated thrice, and sterile water was used as the control. *H. theivora* prefers developing cocoa pods for feeding than younger shoots and leaves (Thube et al., 2019). Moreover, detached cocoa pods retain moisture and turgor pressure longer than other plant parts. Hence, the inoculated insects were maintained on cocoa pods throughout the experiment. Insect mortality was recorded on a daily basis. Mortality data was recorded up to six days after TMBMA1 inoculation as 100% insect mortality was observed in the treatment containing the highest conidial suspension (1×10^8 and 1×10^9 conidia ml^{-1}). Dead adults were incubated in a humidity chamber containing filter paper moistened with sterile distilled water to observe mycosis.

Another assay was performed to know the median lethal time (LT_{50}) of TMBMA1 by using the 1×10^8 conidia ml^{-1} spore suspension against the adults of *H. theivora* as mentioned above. This experiment was repeated thrice, providing confirmatory results. Mortality was recorded every 24 h interval till 100% mortality in treatment. Sterile water was used as the control.

2.7.2. *Field evaluation*

The EPF isolate TMBMA1 was evaluated (1×10^8 conidia ml^{-1}) against *H. theivora* in a farmer field located in Sullia taluk in the Dakshina Kannada district (Karnataka, India) during 2019. The field was located 94 m above mean sea level, with coordinates 12°33′ N 75°22′ E. A total of 360 cocoa trees were used for the experiment. The experiment was laid out in a randomized block design (RBD) with four replications in four different blocks (15 trees/replication; 60 trees/treatment). The field evaluation of TMBMA1 was done along with one recommended insecticide and three biopesticides. The experiment was conducted on an 11 year old cocoa plantation. Details of the insecticides, commercial biocontrol formulation and other bio-pesticides used are shown in Table 1. The treatments were labelled as follows: T_1 = Lambda cyhalothrin 5EC, T_2 = Neem oil 1000 PPM, T_3 = *Metarhizium anisopliae* TMBMA1, T_4 = *Metarhizium anisopliae* (Commercial formulation), T_5 = Horticultural Mineral Oil, T_6 = Untreated control. The tea mosquito bug is reported to feed on flower buds, young cherelles, developing pods, and newly emerged cocoa shoots. Hence, spraying was aimed to cover the entire canopy of trees from all the treatments. The mean quantity of spray solution required to cover the entire canopy of one cocoa tree was calibrated by spraying the water solution on fifty trees using a gator rocker sprayer (American Spring & Pressing Works Pvt. Ltd., Mumbai, India). The volume of spray solution for covering the entire canopy of the tree was standardized as one litre per plant. Each treatment was imposed twice in a season. The first application was made during the first fortnight of October 2019, when mirid infestation was initiated under field conditions, and the next spray was applied 45 days later. The untreated control consisted of water without any additives or active substances.

Table 1
Details of treatments evaluated under field conditions.

Treatment	Particular	Dose	Manufacturer
T1	Lambda cyhalothrin 5EC	1 ml/litre	Syngenta India Ltd., India
T2	Neem oil 1000 PPM	5 ml/litre	Agro Bio-Tech Research Centre Ltd., India
T3	*Metarhizium anisopliae* TMBMA1	1×10^8 conidia/ml	New isolate
T4	*Metarhizium anisopliae*	1×10^8 conidia /ml	Agro Bio-Tech Research Centre Ltd., India
T5	Horticultural Mineral Oil	1 ml/litre	Krishna Antioxidants Pvt. Ltd., India
T6	Control (Sterile water)		–

Observations on mirid infestation (healthy and infested pods per treatment) were recorded at one-month intervals till the pods attained maturity. Effects of the treatments on the severity of the infestation were recorded based on the mean number of feeding lesions in a 2 cm^2 area per pod per treatment (Suppl. Fig. 1). Forty pods per treatment (ten pods per replication) were recorded. The ability of the EPF to infect and cause sporulation under field conditions in treated blocks was confirmed by random observation of insect cadavers and re-isolation of the mother culture at ten day intervals until harvesting of the pods. Conidial germination of the suspension prepared (5 ml sterile distilled water per insect cadaver) out of pre-incubated insect cadavers (N = 11) was assessed by direct microscopic observation at 400 X magnification; germinated conidia were identified as those with germ tubes longer than the width of an ungerminated conidium (Faria et al., 2015; Yeo et al. 2003). The whole experiment was repeated in the year 2020, by initial imposing of the treatments during the last fortnight of September when mirid infestation was noted on younger shoots. All intercultural operations were performed as per the standard recommended practices (Apshara et al., 2018).

2.8. *Statistical analysis*

Percentage data were normalized using arcsine transformation, and numerical data were square-root transformed before analysis. The analysis was conducted on the transformed data, and only the untransformed data is presented. The median lethal time (LT_{50}) of TMBMA1 was calculated using Polo Plus software (LeOra Software, Petaluma). The data on the mortality of *H. theivora* under laboratory bioassay and the effect of various treatments over the severity of infestation under field conditions were subjected to one-way ANOVA using SPSS software (version 18.0; SPSS, Chicago, IL, USA). When the year wise data on mirid incidence was subjected to analysis of variance (ANOVA), it revealed homogenous subsets [ratio of error mean square of two year (1.94) was less than calculated table F value (2.69)], therefore, the data from the repetitive experiments was subjected to combined ANOVA to assess the effect of year, block within the year, treatments and their interaction on mirid incidence. When ANOVA was significant, comparisons of means were made using Tukey's post hoc test at the 5% significance level ($P < 0.05$).

3. Results

3.1. Morphological characterization

TMBMA1 colonies showed a slow growth rate (6 mm day^{-1}) and covered 90-mm Petri plates in 14 days at 28 ± 2 °C. Fungal culture was whitish to pale green initially and became dark green after sporulation. Colonies were dense, dusty, and showed typical concentric rings of dark green conidia intermingled with whitish mycelium (Fig. 2a). The reverse side of colonies was pale yellow with a smooth surface. Conidiophores are highly branched with short cylindrical-shaped phialides (Fig. 2b). The conidia were oval, ellipsoid to rod-shaped, aseptate with 5.75 ± 0.09 μm length X 2.33 ± 0.06 μm width (Fig. 2c). Based on the detailed morphological characteristics, the National Fungal Culture Collection of India, Agharkar Research Institute, Pune identified the isolate TMBMA1 as *Metarhizium anisopliae*.

3.2. Molecular characterization and phylogenetic analysis

The ITS rDNA sequence (559 bp) generated from the isolate TMBMA1 was deposited in the NCBI GenBank under the accession number MT229077. BLAST analysis of the sequences revealed 100% similarity with EPF *M. anisopliae* (Accession no. GQ888737) isolated from a dead cadaver of *Rhynchophorus ferrugineus*. In the phylogenetic analysis with the other species of the *Metarhizium* genus, we found that TMBMA1 grouped within the *M. anisopliae* clade (Fig. 3). Thus, the molecular and phylogenetic analyses of the ITS rDNA sequence of isolate TMBMA1 confirmed its identity as *M. anisopliae,* supporting our morphological characterization.

3.3. Laboratory bioassays

The inoculation of conidial suspension of TMBMA1 to the adults of *H. theivora* lead to significantly high insect mortality within six days of inoculation (Table 2). A significant difference ($F_{7,24} = 54.47$; $p < 0.0001$) in the insect mortality was observed among the various concentrations used. Insect mortality varied between 17.5% and 100% at different concentrations (Table. 2). Higher conidial suspensions (1×10^8 and 1×10^9 conidia ml^{-1}) proved to be the most virulent with 100% insect mortality at six days of inoculation. The survival rate of the insect population inoculated with 1×10^8 conidia ml^{-1} reduced to 50% within 3 days of inoculation ($F_{5,24} = 129.89$; $p < 0.0001$) and no insects survived after 6 days (Fig. 4). Hence, the highly effective conidial suspension of 1×10^8 conidia ml^{-1} was further tested for median lethal time (LT_{50}) analysis and for field evaluation. The LT_{50} of TMBMA1 against adults of *H. theivora* at 1×10^8 conidia ml^{-1} conidial suspensions was 3.01 days with a lower limit of 2.76 and an upper limit of 3.30 days. Green muscardine coating over the dead insect cadaver was observed after four days of incubation in a humidity chamber (Fig. 5a). Post mortality dissection of the insect showed the presence of numerous spores of TMBMA1 inside the body (Fig. 5c) and growth of conidiophores on the insect cuticle (Fig. 5d).

3.4. Field evaluation

In line with the laboratory bioassay results, we observed that the EPF isolate TMBMA1 was highly effective against mirid *H. theivora* in the field (Fig. 6). The analysis of the data from the field experiments showed non-significant effect of the year ($F = 1.791$, df = 1, 20, $p = 0.196$), block within the year ($F = 0.777$, df = 4, 20, $p = 0.553$) and year and treatment interaction ($F = 2.188$, df = 5, 20, $p = 0.096$) on mirid severity. However, the effect of treatments significantly affected the mirid severity ($F = 69.451$, df = 5, 20, $p < 0.001$). Among the treatments, the lowest mirid incidence was observed in the treatment with Lambda-cyhalothrin (severity: 0.85) which was not-significantly different from the treatment with TMBMA1 (severity: 1.71). The commercial formulation of *M. anisopliae* (Manufacturer: Agro Bio-Tech Research Centre Ltd., India) was showed a significantly higher pest incidence (severity: 11.70) as compared to TMBMA1. However, the highest mirid severity was observed in the untreated control (severity: 13.98) (Fig. 6). Dead cadavers with a fungal mat over the bodies were recovered from TMBMA1-treated trees even up to 40 days after spraying (Fig. 5b). The isolate TMBMA1 showed high potential to infect and cause sporulation under field condition with higher mean conidial germination (96.27 ± 0.84 %) for both the years.

4. Discussion

The role of agrochemicals in crop protection is significant in terms of yield improvement and a better economy. However, their widespread and often reckless use has resulted in pest resistance, the resurgence of secondary pests, and disruption of natural enemy complexes, thus reducing the efficacy of natural control processes and ecosystem health (Mantzoukas and Eliopoulos, 2020). These factors, coupled with concerns about the impact on the environment and human safety, have provided the momentum to develop more environmentally safe strategies that are cost-effective, pest-suppressive, and reliable. Moreover, as cocoa is a food crop (used for the preparation of chocolate, butter, cocoa powder, etc.), the use of broad-spectrum insecticides should be the last resort for pest and disease management. Mirids are the major insect pest of cocoa with 75% yield reduction potential reported in India (Thube et al., 2019). Hence, the output of the present study would be a boon for the cocoa growers and provide a more cost-effective, eco-friendly and host-specific pest combating option for sustainable management of

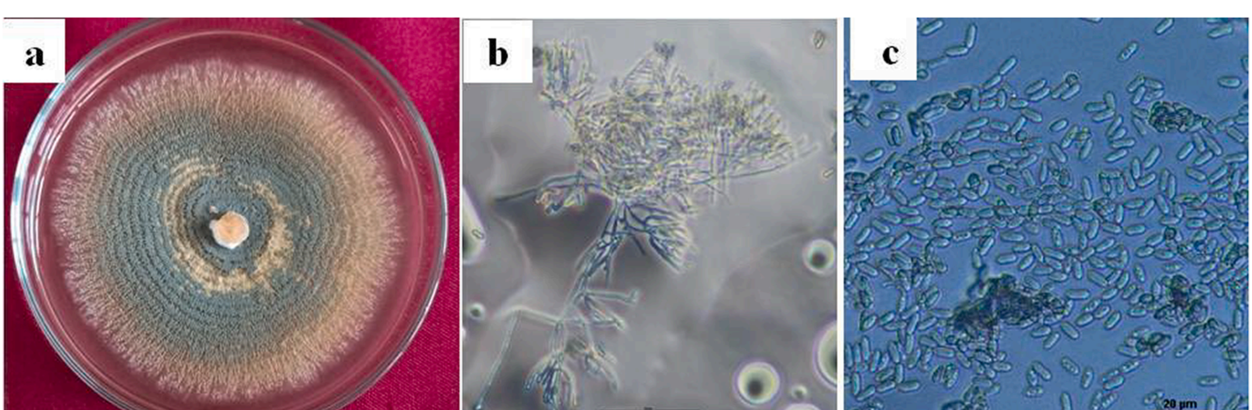

Fig. 2. Morphological characteristics of *Metarhizium anisopliae* TMBMA1. a Dark green colony with concentric rings. b Profusely branched conidiophores. c Rod shaped, aseptate conidia.

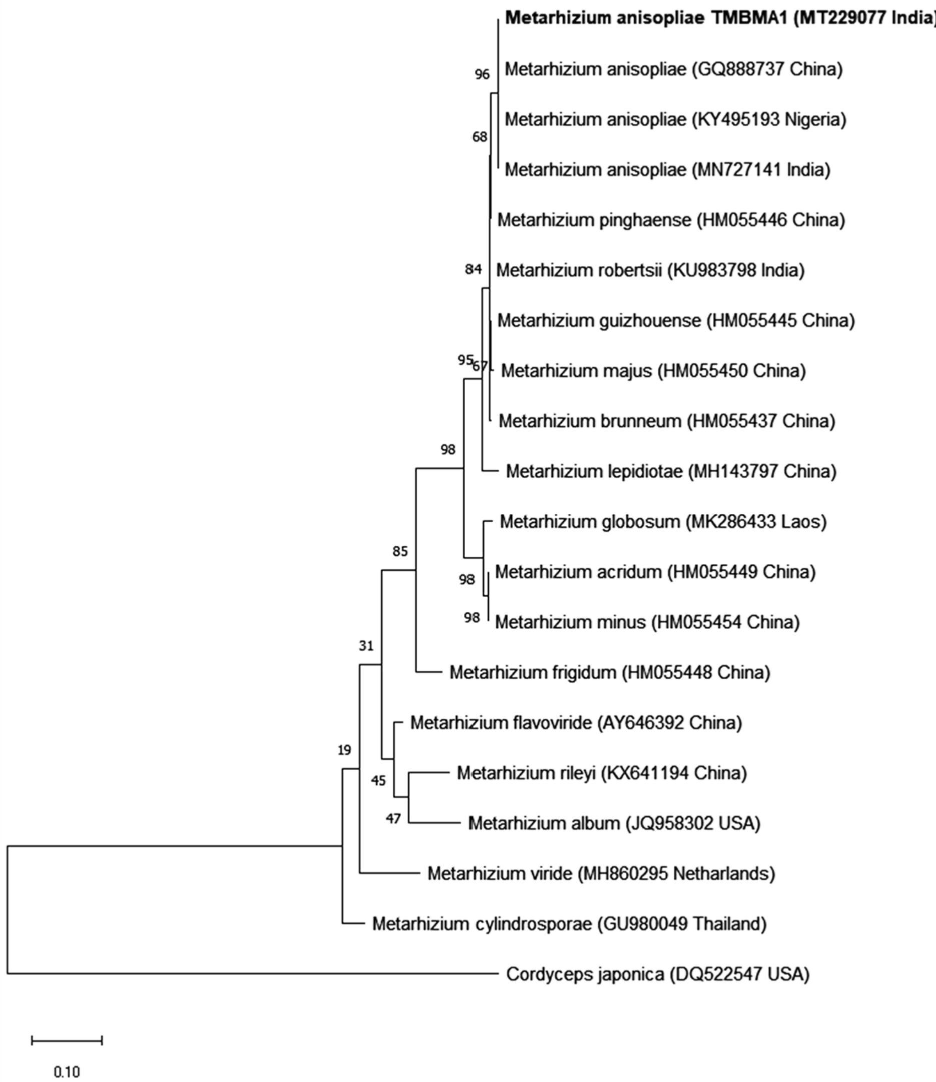

Fig. 3. Phylogenetic relationship of *Metarhizium anisopliae* TMBMA1 with closely related *Metarhizium* species, based on the ITS rDNA sequences. The analysis was based on the maximum likelihood (ML) algorithm using the Kimura 2-parameter model, with bootstrap support estimated from 1000 repetitions. *Cordyceps javanica* was used as outgroup taxon.

Table 2
In-vitro efficacy of various concentrations of *Metarhizium anisopliae* TMBMA1 against *H. theivora* adults six days post inoculation. Different lowercase letters indicate statistically significant differences ($P < 0.05$, Tukey's HSD test).

Dose (conidia /ml)	Mortality (%±SE)
1×10^3	$17.5^{ef} \pm 4.79$
1×10^4	$37.5^{de} \pm 6.29$
1×10^5	$57.5^{cd} \pm 8.53$
1×10^6	$75^{bc} \pm 5.00$
1×10^7	$92.5^{ab} \pm 4.78$
1×10^8	$100^{a} \pm 0.00$
1×10^9	$100^{a} \pm 0.00$
Control	$10^{f} \pm 0.00$

mirids in cocoa, compared to chemical insecticides.

Fungi are the most common insect pathogens and are well suited for development as potential bioagents with ease in mass production protocols (Thomas and Read, 2007). The efficacy of EPF mainly depends on the native conditions they originated from, as they are well adapted to that ecosystem. The infection potential of the EPF in real field conditions also greatly depends on the relative humidity prevalent in the field. The self-perpetuation rate of any EPF is directly proportional to the prevailing humidity during the sporulation phase. The EPF isolate TMBMA1, identified and evaluated in the present investigations in alignment with all aforesaid features, proved effective against the mirid *H. theivora* not only under controlled laboratory conditions but also in a cocoa plantation in real field conditions. The Dakshina Kannada district, where the field experiment was conducted, covers an area nestled in between the Western Ghats to its east and the Arabian Sea to its west. The average annual rainfall and humidity of this district are 4,030 mm and 75%, respectively. Relative humidity records its peak (greater than 90%) during the monsoon season (June-September). High rainfall and humidity were the most important reasons for the synergistic effect of TMBMA1 in controlling the population of mirids in cocoa.

Most cropping systems and their insect pests are transient, being present for only one or two growing seasons. In addition, widespread adoption of crop rotation on large areas of monoculture creates a very unstable environment for any EPF to establish in that habitat (Jaronski, 2010). Annual disruption in a habitat not only removes the insect hosts but in many cases directly destroys the microbial agent. Inundation with a microbial agent, sometimes repeatedly, is therefore necessary. Unlike

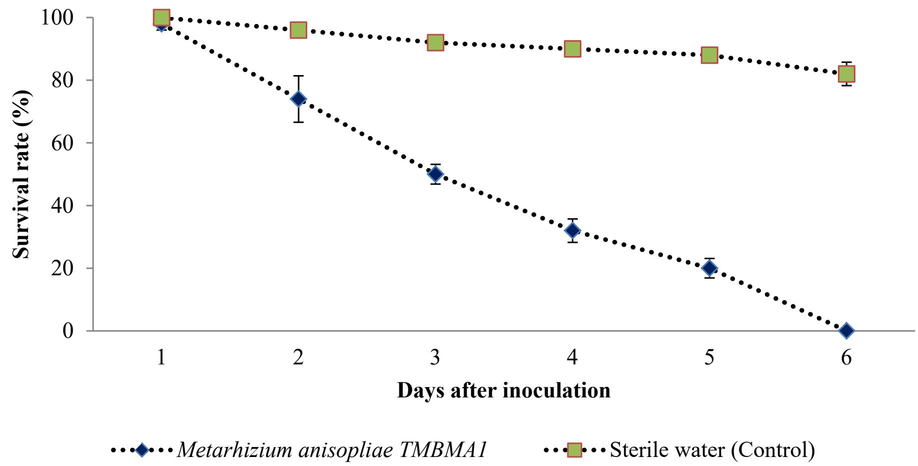

Fig. 4. Percent survival rate of *Helopeltis theivora* after *Metarhizium anisopliae* TMBMA1 inoculation with 1 × 10^8 conidia ml^{-1}.

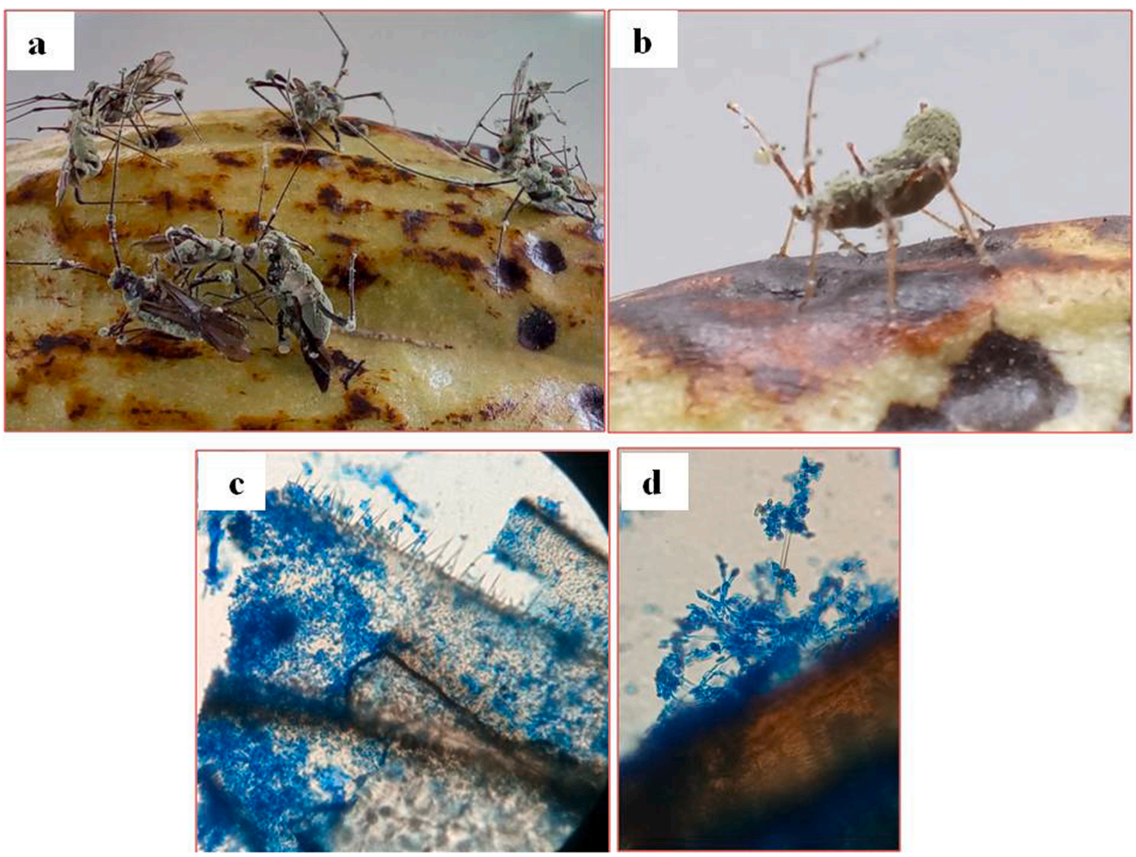

Fig. 5. Entomopathogenicity of *Metarhizium anisopliae* isolate TMBMA1 against *H. theivora*. a Mortality of inoculated adults. b. Mortality of inoculated nymph recovered from the field. c. Sporulation inside the dead cadaver. d Growth of conidiophores over the cuticle of the dead cadaver.

these unstable habitats, cocoa is an integral part of the perennial palm-based cropping system in Peninsular India, with negligible modulation in the system. In major cocoa growing African countries, it is an important component of the agroforestry cropping system. Moreover, under Indian conditions, mirid populations appear during September and attain their peak during March-April. The off-season population survives on young tender shoots and alternative weed hosts in the absence of cocoa pods. The presence of an undisturbed perennial cropping system, continuous availability of a susceptible insect host, and favourable climatic conditions would contribute to the success of TMBMA1 in the sustainable management of mirid populations in cocoa.

Virulence is one of the important traits of a good biological control agent (Mascarin et al., 2013). The EPF isolate TMBMA1 was found highly virulent to the adults of *H. theivora* with a LT_{50} of 3.01 days. In line with our results, Tuncer et al (2019), reported an LT_{50} of 4.43 days against invasive ambrosia beetle, *Xylosandrus germanus* using the native isolate of *M. anisopliae* with 1 × 10^8 conidia ml^{-1} conidial suspensions. Similarly, a native isolate of *M. brunnum* was found effective against neotropical brown stinkbug *Euschistus heros* (F.) with an LT_{50} of 6 days (Resquín-Romero et al., 2020). However, TMBMA1 proved more virulent with the reduction in LT_{50} by 32 times (3.01 days) with the same conidial concentration.

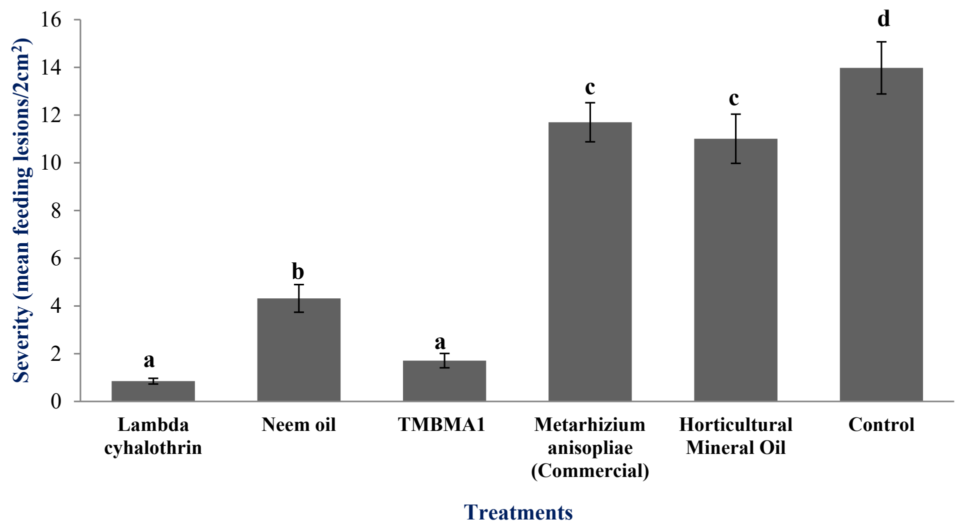

Fig. 6. Field efficacy of *Metarhizium anisopliae* TMBMA1 with other commercial insecticides against mirid in cocoa. Different letters on error bars indicate significant differences between treatments ($P < 0.05$, Tukey's HSD test).

The real success of any biocontrol agent is mostly dependent on its performance under field conditions. TMBMA1 evaluated under cocoa plantation was found potent with low severity of mirid infestation (1.71) compared to the control (13.98). Nevertheless, the severity of infestation in TMBMA1 sprayed plots is significantly higher (1.71) than after treatment with chemical insecticides (0.85). This difference in severity can be further minimized by increasing the conidial suspension during spraying. When compared to the commercial strain of *M. anisopliae*, TMBMA1 was found ten times more effective than the marker standard. Such a rapid mortality rate is extremely rare among entomopathogenic fungi, suggesting that this isolate could be developed into an effective biological formulation for managing mirids in the cocoa ecosystem, in an eco-friendly manner without any setback to the plantation system biodiversity. To our knowledge, this is one of the most potent bioagents proved effective in the bio-suppression of cocoa mirid and first of its kind documented so far. Therefore our study recommends the use of TMBMA1 in cocoa growing areas of southern India at 1×10^8 conidia ml^{-1} concentration during September and November for effective management of TMB.

In summary, *M. anisopliae* isolate TMBMA1 shows great potential for controlling *H. theivora* in the cocoa plantations of Southern India; our hypothesis was confirmed as the mirid severity in cocoa was significantly lower than in the control and other bio-molecules tested. Given the rapid mortality of the mirid population caused by TMBMA1 and its high efficacy under field conditions, this isolate has a high potential to be developed into a potent biological formulation for sustainable management of this polyphagous pest. Further, its efficacy against other species of mirid (*H. bradyi* and *H. antonii*) in alternate crops like tea and cashew could be evaluated.

CRediT authorship contribution statement

Shivaji Hausrao Thube: Conceptualization, Methodology. **R. Thava Prakasa Pandian:** Conceptualization, Methodology. **Merin Babu:** Conceptualization, Methodology. **A. Josephrajkumar:** Conceptualization, Methodology. **Priyank H. Mhatre:** Software. **P. Santhosh Kumar:** . **B.J. Nirmal Kumar:** . **Vinayaka Hegde:** Supervision. **Satish Namdeo Chavan:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2022.104909.

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