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DNA barcoding reveals the natural occurrence of *Beauveria bassiana* (Balsamo) Vuillemin in two-spotted spider mite, *Tetranychus urticae* Koch in Bhendi [*Abelmoschus esculentus* (L.) Moench] ecosystem in Coimbatore district of Tamil Nadu

V. P. Abarna* and R. Vishnupriya

Abstract

Background: Two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae) incidence is a major constraint in vegetable cultivation. The indiscriminate use of acaricides is causing environmental threat and also residual effect in vegetables. To develop an eco-friendly management strategy, an investigation was made to access the natural occurrence of entomopathogenic fungi (EPF) infecting mites in bhendi, India ecosystem.

Results: Natural incidence of EPF was high during the month of December. Twelve EPF isolates were isolated from the mycosed *T. urticae* cadavers using Potato Dextrose Agar Medium. Morphological studies confirmed that the isolated fungi were *Beauveria bassiana*. PCR amplification of ITS region was carried out and the results showed, amplification at 560 bp. In NCBI database, the sequence of the virulent isolates had shown 99.2, 98.7, 99.1, 97.7 and 96.8% homology with other *B. bassiana* isolates, confirming the occurrence of *B. bassiana* mycosed mites in vegetable ecosystem.

Conclusion: The isolate MZ749636 (*B.b-7*) was found to be virulent against *T. urticae* causing 86% mortality of *T. urticae* at the conidial load of 1×10^8 conidia/ml at laboratory conditions and so it could be utilized for the eco-friendly management of *T. urticae*.

Keywords: *Beauveria bassiana*, Characterization, Pathogenicity, *Tetranychus urticae*

Background

Bhendi [*Abelmoschus esculentus* (L.) Moench], a warm vegetable crop, grown over 509.0 million hectares in India. Among the biotic constraints in cultivation of bhendi, sucking pests like the mite (*Tetranychus urticae* Koch), aphid (*Aphis gossypii* Glover) and whitefly

(*Bemisia tabaci* Gennadius) are considered important (Chinniah 2000). Prasad and Singh 2011, reported nearly six different mite species, including two-spotted spider mite (*T. urticae* Koch), Red spider mite (*T. macfarlanei* Baker and Pritchard), Red spider mite (*T. ludeni* Zacher), false spider mite (*Brevipalpus phoenicis* Geijskes), Broad russet mite (*Polyphagotarsonemus latus* Banks), and tomato russet mite (*Aceria lycopersici* Wolfenstein). Among these, *T. urticae* pose a major threat to bhendi cultivation, especially during the summer, spring and post-rainy

*Correspondence: abarnavijayakumar140@gmail.com

Department of Agricultural Entomology, TNAU, Coimbatore-3, Tamil Nadu, India

seasons, leading in severe yield loss of 7 to 48% (Roseleen and Ramaraju 2010). *T. urticae* also infests the important vegetable crops in the region.

In India, *T. urticae* had been recorded in more than 900 hosts including bhendi, brinjal, cucumber, potato etc., (Migeon et al. 2010). Due to short life span of 9–10 days and high reproductive potential of more than 100 eggs/female, it had progressed to the position of a major pest of vegetables with a broader host range (Miyazaki et al. 2013).

Insecticides and acaricides are the preferred management tools in view of accessibility and quick control against phytophagous mites. However, acaricides are employed indiscriminately, leading in the development of acaricidal resistance (Simma et al. 2020). Therefore, non-chemical alternative methods have been explored for managing *T. urticae*.

Insect pathogens, particularly entomopathogenic fungi (EPF) have been employed to manage a variety of insect pests, particularly mites with great effectiveness. These EPF are known to infect the pests naturally leading to the death due to mycosis. *Beauveria bassiana* (Balsamo) Vuillemin (Jeyarani et al. 2011), *Metarhizium anisopliae* (Meetschnikoff) (McGuire and Northfield 2020) and similar EPF were known to cause natural infection on *T. urticae*. There lies a greater opportunity for integration of these EPF in applied biological control of two-spotted spider mites. Several authors have reported the variable levels of virulence of EPF against target pests in laboratory and field investigations. Hence, the present study aimed to find the diversity of various EPF causing infection on two-spotted spider mites, and exploiting them for eco-friendly management.

Methods

Survey and collection of samples

Survey was conducted in Coimbatore district of Tamil Nadu viz., TNAU Orchard (11.0122° N, 76.9354° E), TNAU insectary (11.02232° N, 76.9434° E) Thondamuthur (10.9899° N, 76.8409° E), Narasipuram (10.9880° N, 76.7740° E), Aandipatti (10.0015° N, 77.6164° E), Pichanur (10.8623° N, 76.8727° E), Theethipalayam (10.9694° N, 76.8956° E), Madampatti (10.9698° N, 76.8598° E), and Poosaripalayam (11.0062° N, 76.8668° E) during 2020–2021 to isolate the EPF infecting two-spotted spider mites. The mites were examined in the field using a 40- \times magnifying hand lens and the mite cadavers showing natural external growths of fungi (Jeyarani et al. 2011) were collected in a 1.5 ml sterile eppendorf tube using a camel hair brush (000 size) and further investigations were carried out in the AICRIP- Acarology Laboratory, Department of Agricultural Entomology, TNAU, Coimbatore. The mites showing fungal infection were placed

in a Petri plate layered with sterile moistened filter paper and incubated at 26 ± 2 °C for further mycelial development and sporulation (Goettel and Inglis 1997). Mites were also collected throughout the survey to confirm the species of two-spotted spider mite. The collected mites were mounted on the slide using the Hoyer's Medium. Morphological identification of the species was made using 40–100 \times phase contrast microscope (Euromex iscope, USA).

Isolation of entomopathogenic fungi

Potato Dextrose Agar Medium (PDA) (Dinu et al. 2012) was used for the isolation of the EPF. Fungus with white powdery conidial mass on infected two-spotted spider mites were collected and kept at temperature of 25 ± 2 °C and relative humidity of 85–90%t under laboratory conditions for further sporulation. The sporulated two-spotted spider mites were surface sterilized, using 70% ethanol for a minute, followed by 0.1% sodium hypochlorite for a second and rinsed using distilled water for four to five times and the conidia from the infected specimen's surface were transferred to a 90 mm Petri plate containing PDA medium and were incubated at an ambient temperature for sporulation (Abdo et al. 2008). Sub-culturing was made by inoculating a single colony into 90 mm Petri plate containing PDA medium and incubated at a 25 ± 2 °C and RH 85–90% for two weeks in order to obtain the pure culture.

Morphological characterization

To study the morphological characters of the isolates, 15-day old culture was used (Malarvannan et al. 2010). The morphological characters, such as colony colour, mycelial character and spore structure were studied by phase contrast microscopy according to Samson et al. 2013.

Molecular characterization

The CTAB (cetyl-trimethyl ammonium bromide) method was used to extract genomic DNA as described by Zhang et al. (1925). Using a cork-borer, a single colony from the mother culture was inoculated into a 250 ml flask containing 100 ml Potato Dextrose Broth (PDB). For 1–2 weeks, the liquid culture was shaken at 250 RPM at 28 °C (Reddy et al. 2016). To separate mycelial mat and culture filtrate, the cultures in the flasks were aseptically filtered through Whatman No 1 filter paper (Viswanath et al. 2008). The mycelial mat was separated from the broth was dried on the Whatman no 1 filter paper for 2 h. One hundred mg of the mycelial mat was ground using 800 μ l of 2% CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 20 Mm EDTA, 100 Mm Tris-HCl, 1% PVP and 0.2% mercaptoethanol with the pH of 8) (Ashwathi

et al. 2017). It was then transferred to a sterile Eppendorf tube and incubated for 1 h at 65°C in a water bath. An equal volume of phenol:chloroform:iso amyl alcohol (25:24:1) was added and spun at 10,000 rpm in refrigerated centrifuge for 10 min at 4 °C. After centrifugation, the supernatant was transferred to another Eppendorf tube containing an equal volume of ice cold iso-propanol and incubated overnight at –20 °C. Later, it was spun at 13,000 rpm for 10–15 min at 4 °C and supernatant was discarded after centrifugation and the pellet was washed with 70% ethanol and dried. The resultant pellet was then suspended in 30 µl of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) (Chaithra et al. 2020). The presence of DNA was confirmed using a Nano drop Spectrophotometer (Thermo Scientific Company, USA). 1 µl deionized water was placed on the bottom optical surface for blanking, and the lever arm was closed to guarantee the upper pedestal makes contact. After blanking, 1 µl of the sample was dispensed on the lowest pedestal and the lever arm was closed and the measurement was made as per the procedure by Desjardins and Conklin (2010).

PCR amplification- ITS region

The isolated DNA was amplified using a Polymerase Chain Reaction (PCR). The ITS-1(ITS1-F 5'-TCGGTA GGTAGGTGAACCTGCGG-3') and ITS-4 (18s-ITS4 -R 5'-CAGGAGACTTGTACACGGTCCAG-3') region were amplified using the method described. The PCR product consisted of 5 µl of reaction mixture (200 µM of each dNTP, 3.75 mM MgCl₂ and 0.05 U of Taq DNA polymerase in 20 mM of pH 8.4 Tris HCl containing 50 mM KCl), 2 µl of nuclease free water, 1 µl of DNA and 1 µl each of forward and reverse primer. A negative control (without DNA) was included. The amplification temperature was set to 1 cycle of initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s followed by elongation at 72 °C for 1 min each of 35 cycles and 1 cycle of final elongation at 72 °C for 10 min. The PCR product was subjected to 1% agarose gel electrophoresis stained using ethidium bromide solution of 10 µg/ml and visualised under UV transilluminator. The PCR product was then sent for sequencing to Eurofins Genomics, Bengaluru. The nucleotide sequence obtained was aligned for its similarity using the BLAST software (Islam et al. 2017).

Rearing of two-spotted spider mites, *T. urticae* for pathogenicity testing

The mites collected from the field were reared in the Acarology Laboratory using freshly detached mulberry leaves. The leaves were placed in Petri plates with moistened sterile cotton. Collected mites were released in the Petri plate and allowed to multiplication. The cotton

pads were moistened daily and the mulberry leaves were changed whenever it is necessary (Hassan et al. 2019). After multiplication of the mites, it was transferred to the caged potted bhendi (Bhendi hybrid COBh H 1) plants maintained at Insectary, Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu for further multiplication.

Pathogenicity of the isolated entomopathogenic fungi isolates against *T. urticae*

For testing the pathogenicity of EPF, fully sporulated (15 days old) culture were used (Malarvannan et al. 2010). With a sterile surgical knife, the fungal surface was scraped with 10 ml of sterile distilled water containing 0.01% Tween 80 as a wetting agent (Rombach et al. 1986). The spore concentration was found using the Neubaur haemocytometer. The desired spore concentration (1×10^8 conidia/ml) was prepared by diluting the spore suspension with sterile distilled water.

Mulberry leaf discs were cut to the size of 5 cm diameter and they were dipped in the prepared EPF suspensions (1×10^8 conidia/ml) and dried over a filter paper at the temperature of $25 \pm 1^\circ\text{C}$ and allowed to air dry. Mulberry leaves dipped in the sterile water containing 0.01% Tween 80 served as a control. The treated leaves were placed in Petri plates with moist sterile filter paper and 30 uniform aged adult mites were released to each Petri plate (Lee et al. 1997). The experimental setup was replicated thrice. The mortality of mites was calculated on 24 h interval and percentage mortality was calculated by the formula:

$$\begin{aligned} \text{Percent mortality (\%)} \\ &= \frac{\text{No. of mites died}}{\text{Total no. of mites in each replication}} \times 100 \end{aligned}$$

The percentage reduction over the control was calculated using the formula,

$$\begin{aligned} \% \text{ reduction over control} \\ &= \frac{\% \text{ mortality in treatment} - \% \text{ mortality in control}}{\% \text{ mortality in treatment}} \\ &\times 100 \end{aligned}$$

The dead mites were kept in a separate Petri plates with sterile moist filter paper to allow the fungus to grow on the surface of the cadavers and re-isolation of the pathogen was done to confirm the death due to EPF infection.

Statistical analysis

Mortality days after inoculation (DAI) were adjusted using Abbott's formula (Abbott 1925). Analysis of variance was used to examine the differences in mortality between the fungal isolates and the control group

(ANOVA). The statistical software SPSS 16.0 was used to perform all of the analyses.

Results

Survey and morphological identification of two-spotted spider mites

The identity of live spider mite specimens that were collected during the survey was confirmed based on the morphological characters described by Seeman and Beard (2011). In this study, slides for both male and female mites were prepared and examined. The genital

apertures, striations were the primary parameters considered. The present study revealed the occurrence of *T. urticae* in bhendi. The mycosed dead mites were collected from various locations (Fig. 1) and the per cent infection was calculated (Table 1) and it was maximum (8.42%) at TNAU Orchard. A total of 12 isolates were obtained and they were used for the morphological and molecular confirmation in the present studies.

Identification of EPF isolates

Identification of EPF isolates were done according to Humber (1997). Morphological aspects such as: colony color, colony structure, mycelial texture, conidial shape (Figs. 2, 3) and size were examined under the phase contrast microscope and tabulated (Table 2). The morphological identification revealed that all the isolates produced whitish colony or yellowish white colony with hyaline and septate mycelium and the conidia is either globose or sub-globose indicating that all the isolates were *B. bassiana*. The conidial size of all the isolates were in the range of 2.56–1.95 μm, which further confirms that all the isolates were *B. bassiana*.

Molecular characterization

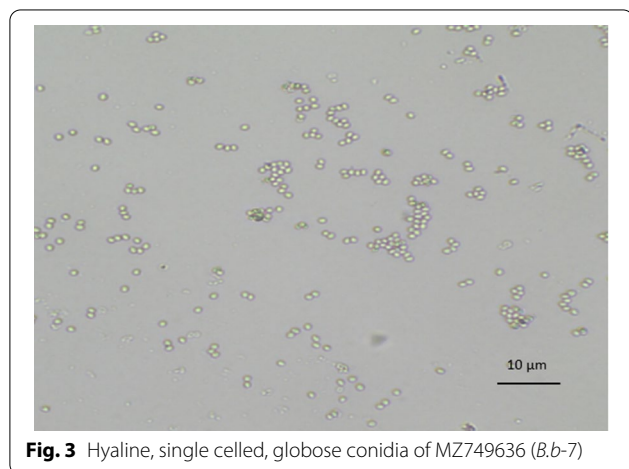
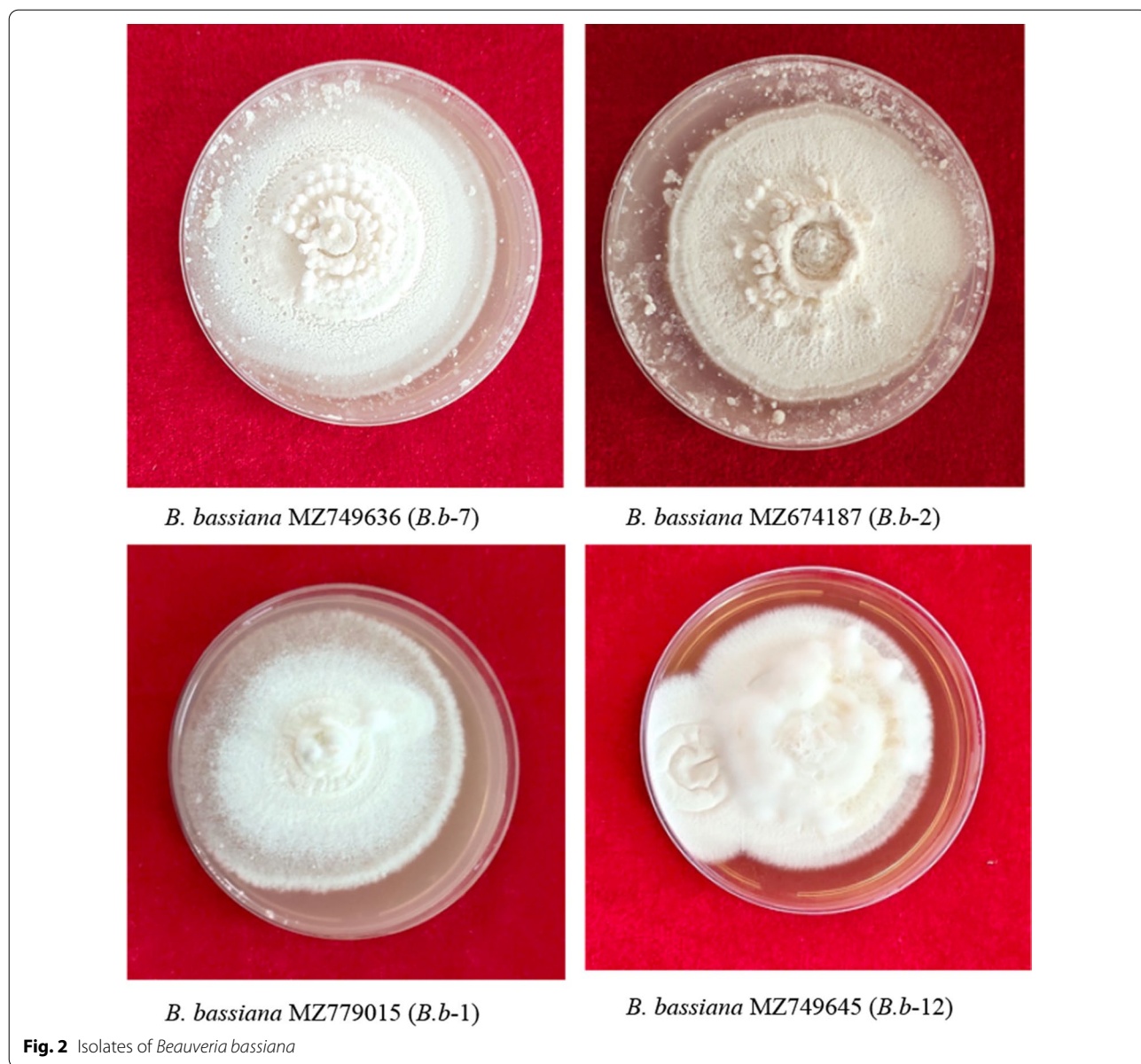
PCR amplification revealed that the presence of the amplification of ITS region in 560 bp, which was used for further confirmation of *B. bassiana*. The PCR products were sent for sequencing to Eurofins Genomics,



Fig. 1 White cottony growth of entomopathogenic on the dorsal sides of *Tetranychus urticae*

Table 1 Survey and incidence of entomopathogenic fungi infestation on two-spotted spider mite, *Tetranychus urticae* in Bhendi

S. no.	Crop variety/hybrid	Host mite species	Place	Location	Per cent mite incidence (%)	Total mites collected	No. of mycosed mites	Mycosed mites (%)
1	Bhendi hybrid COBh H 1	<i>Tetranychus urticae</i>	TNAU orchard	11.0122° N, 76.9354° E	90	73.6	6.2	8.42
2	CO-3- bhendi	<i>T. urticae</i>	TNAU Insectary	11.02232° N, 76.9434° E	100	154	9.8	6.38
3	Bhendi hybrid COBh H 1	<i>T. urticae</i>	Thondamuthur	10.9899° N, 76.8409° E	75	98	7	7.14
4	CO-1-Bhendi	<i>T. urticae</i>	Narasipuram	10.9880° N, 76.7740° E	65	137.8	6.8	4.96
5	Bhendi hybrid COBh H 1	<i>T. urticae</i>	TNAU Orchard	11.0122° N, 76.9354° E	70	106.2	7	6.59
6	CO-3-bhendi	<i>T. urticae</i>	Aandipatti	N 10.0015° E 77.6164°	65	95.8	5	5.21
7	CO-1-Bhendi	<i>T. urticae</i>	Thondamuthur	10.9699° N, 76.8411° E	80	182	9	4.94
8	Bhendi hybrid COBh H 1	<i>T. urticae</i>	Pichanur	10.8623° N, 76.8727° E	85	114.4	7	6.11
9	Bhendi hybrid COBh H 1	<i>T. urticae</i>	Theethipalayam	10.9694° N, 76.8956° E	85	79.6	6.2	8.42
10	Bhendi hybrid COBh H 1	<i>T. urticae</i>	Madampatti	10.9698° N, 76.8598° E	65	74.2	4	5.39
11	CO-3-Bhendi	<i>T. urticae</i>	Poosaripalayam	N 11.0062° E 76.9333°	95	119.8	5.8	4.81
12	CO-1-Bhendi	<i>T. urticae</i>	Madampatti	10.9688° N, 76.8668° E	75	195.8	3	1.53



Bengaluru and the sequence was obtained. The sequence of the effective isolates such as *B.b-7*, *B.b-2*, *B.b-1*, *B.b-12* and *B.b-4* were submitted to NCBI which had 99.2, 98.7, 99.1, 97.7 and 96.8% homology with the other isolates of *B. bassiana*, respectively. The accession number obtained were MZ749636 (*B.b-7*), MZ674187 (*B.b-2*), MZ779015 (*B.b-1*), MZ749645 (*B.b-12*) and MZ674400 (*B.b-4*).

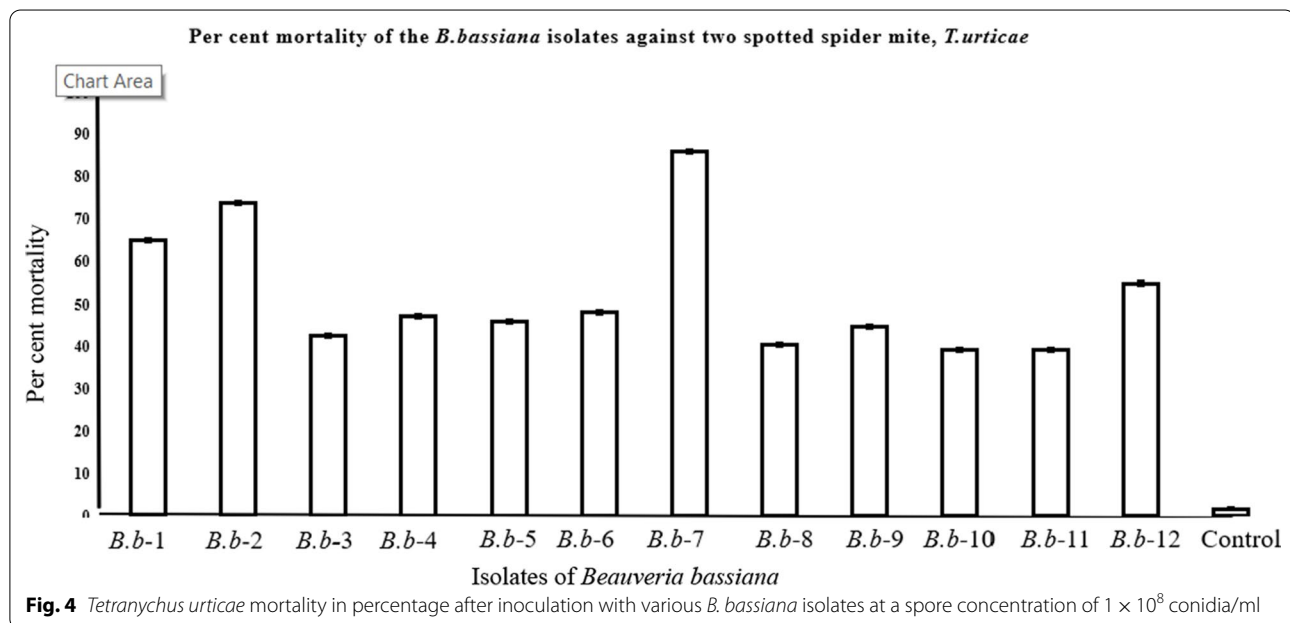
Pathogenicity of EPF and selection of the highly virulent isolates

With a spore concentration of 1×10^8 conidia/ml, the pathogenicity of the 12 *B. bassiana* isolates was tested against the two-spotted spider mite, *T. urticae*. The isolate MZ749636 (*B.b-7*), showed a rapid mycosis in *T.*

Table 3 Efficacy of the entomopathogenic fungi isolates against two-spotted spider mite, *Tetranychus urticae* under laboratory condition tested at the conidial concentration of 1×10^8 conidia/l

Isolates	Mortality of mites/days after inoculation (DAI)				Per cent mortality (%)	Per cent reduction over control
	Mean of three replications					
	3 DAI	5 DAI	7 DAI	9 DAI		
<i>B.b-1</i>	4.50 ^b ± 0.12	6.17 ^{cd} ± 0.06	14.17 ^c ± 0.05	19.50 ^c ± 0.07	65.00	97.44
<i>B.b-2</i>	6.17 ^{ab} ± 0.07	10.83 ^b ± 0.06	17.17 ^b ± 0.05	22.17 ^b ± 0.04	73.90	97.74
<i>B.b-3</i>	4.17 ^b ± 0.08	5.83 ^{cde} ± 0.06	7.83 ^d ± 0.06	12.83 ^{fg} ± 0.04	42.76	96.10
<i>B.b-4</i>	2.83 ^c ± 0.08	5.17 ^{def} ± 0.07	7.83 ^d ± 0.06	14.17 ^{ef} ± 0.04	47.23	96.47
<i>B.b-5</i>	4.83 ^b ± 0.07	6.17 ^{cd} ± 0.06	7.83 ^d ± 0.06	13.83 ^{ef} ± 0.04	46.10	96.38
<i>B.b-6</i>	4.17 ^b ± 0.08	6.50 ^c ± 0.11	9.17 ^c ± 0.05	14.50 ^e ± 0.07	48.33	96.55
<i>B.b-7</i>	6.50 ^a ± 0.11	12.17 ^a ± 0.05	19.83 ^a ± 0.04	25.83 ^a ± 0.03	86.10	98.06
<i>B.b-8</i>	1.83 ^d ± 0.10	4.83 ^{efg} ± 0.07	6.17 ^e ± 0.06	12.17 ^g ± 0.05	40.56	95.89
<i>B.b-9</i>	0.50 ^e ± 0.00	2.83 ^h ± 0.08	3.83 ^g ± 0.08	13.50 ^{ef} ± 0.08	45.00	96.30
<i>B.b-10</i>	0.83 ^e ± 0.12	4.17 ^g ± 0.08	5.17 ^f ± 0.07	11.83 ^g ± 0.09	39.43	95.77
<i>B.b-11</i>	2.83 ^c ± 0.08	4.50 ^{fg} ± 0.12	5.83 ^{ef} ± 0.13	11.83 ^g ± 0.09	39.43	95.77
<i>B.b-12</i>	4.17 ^b ± 0.08	6.83 ^{bc} ± 0.06	9.50 ^c ± 0.09	16.50 ^d ± 0.07	55.00	96.97
Control	0.50 ^e ± 0.00	0.50 ⁱ ± 0.00	0.5 ^h ± 0.00	0.50 ^h ± 0.00	1.67	0.00
CD (0.05)	0.24	0.21	0.20	0.18		
SE(d)	0.12	0.10	0.09	0.08		

No. of insects per replication: 30. Values sharing same alphabets in superscript are statistically on par based on ANOVA at the 5% level of significance



urticae with maximum mortality of 86.10%, followed by the isolate MZ674187 (*B.b-2*), which caused 73.90% mortality. In case of the isolates MZ779015(*B.b-1*), MZ749645 (*B.b-12*) and MZ674400(*B.b-4*) which caused mortalities of 65.00, 55.00 and 47.23% mortality, respectively at 1×10^8 conidia/ml after 12 days of treatment (Table 3). The isolates *B.b-10* and *B.b-11* showed the least mortality of 39.43% (Fig. 4). The mortality of

the mites started third day after EPF treatment and increased up to 12 days after post inoculation. The present study has clearly indicated that the period from 3rd day to 9th day was required for the maximum mortality (86.10%) of the two-spotted spider mite and the infected mites acts as the inoculum for two-spotted spider mite to become infected.

Discussion

Entomopathogenic fungal (EPF) spore adheres to the skin of the insects and later it forms the germination nail and penetrates the epicuticle and reaches hypodermis with the help of protein degrading enzymes. These hyphae after reaching the hypodermis multiply and spread in the body cavity and kill the insect (Altinok et al. 2019). The chlamydospores formed by the fungi germinates to form conidiophores on the skin of the dead insect under appropriate conditions and the spores are ready for new infections (Goettel et al. 2005). The present study was made to explore the diversity of EPF infecting two-spotted spider mite in Coimbatore district, Tamil Nadu. Previous studies were conducted by Jeyarani et al. (2011) in Coimbatore region of Tamil Nadu and recorded 5.25% infection of *B. bassiana* occurred on *T. urticae* in bhendi ecosystem which showed similarity to the present study results as the per cent incidence of *B. bassiana* in Bhendi ecosystem ranged from 1.53 to 8.42%.

Gebremariam et al. (2021) characterized the EPF morphologically indicating the presence of white or yellowish white colony, globose or sub-globose conidia were essential for the confirmation of *B. bassiana*. In the present study, the isolated EPF isolates produced whitish and yellowish white colony, globose and sub-globose conidia were in accordance with the findings of (Gebremariam et al. 2021).

DNA barcoding is very useful for identification of fungal species. The internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene cluster are used as the fungal barcode (Xu 2016). The major advantage of using ITS is making it possible to amplify the gene from small amounts of biological specimens. The entire ITS region was about 600 bp long and contained two variable spacers, ITS-1 and ITS-2 which were separated by the highly conserved 5.8S rRNA gene in fungi (White et al. 1990), which was similar to the present findings. The molecular characterization of *B. bassiana* isolates by Dhar et al. (2019) observed the amplification of ITS region in 524 bp which is in line with the findings of the present study as the amplification of ITS region was observed at 560 bp.

Seiedy et al. (2010) found that the *B. bassiana* isolate DEB1008 caused 74.68% mortality at the spore concentration of 1×10^8 conidia/ml, which is in accordance with the present study as the isolate MZ674187 (*B.b-2*) caused a maximum mortality of 73.90% in laboratory condition. Yanar et al. (2018) reported that the two isolates of F-12 and F-56 caused the maximum mortality of 78.3 and 76.7%, respectively at the conidial concentration of 1×10^8 conidia/ml, which showed similarity to the results of the present investigation. The isolates BGF14

and BCA32 caused 25.88–61.92 and 32.36–62.03% mortality of *T. urticae* at the conidial concentration of 10^8 conidia/ml, which is analogous with the present results (Yucel 2021). At a spore concentration of 1×10^8 conidia/ml in laboratory circumstances, the *B. bassiana* isolates PPRC 19, PPRC 27, PPRC 29, and PPRC 66 were moderately virulent against *T. urticae*, producing 60–80% death, which corresponds to the findings of the present study and the isolates 9614 and 9609 were highly virulent with the mortality range of 86–90% (Negash et al. 2017), which is in further conformity to the present results as the isolate MZ749636 (*B.b-7*) resulted in 86.10% mortality of the two-spotted spider mite.

Conclusion

Under laboratory circumstances, *Beauveria bassiana* isolates MZ749636 (*B.b-7*), MZ674187 (*B.b-2*) and MZ779015 (*B.b-1*) were highly successful in controlling the two-spotted spider mite, *T. urticae*. These three isolates can be utilized in future for effective control in field conditions.

Abbreviations

ANOVA: Analysis of variance; CTAB: Cetyl-trimethyl ammonium bromide; DAL: Mortality days after inoculation; EPF: Entomopathogenic fungi; PCR: Polymerase chain reaction; PDA: Potato Dextrose Agar; PDB: Potato Dextrose Broth.

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Author contributions

The author (VPA) carried out the experiment, recorded data, interpreted the results and wrote the manuscript. The second author (RV) corrected the manuscript. Both authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The author declares that there are no competing interests.

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