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Molecular characterization of new isolates of the entomopathogenic fungus *Beauveria bassiana* and their efficacy against the tobacco caterpillar, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae)

Sushant Dhar, Vikas Jindal*, Monu Jariyal and V. K. Gupta

Abstract

Thirteen fungal isolates, having characteristics similar to *Beauveria bassiana*, were isolated from field soils of Punjab, India, on *Beauveria*-specific selective medium (BS medium). Molecular screening of the *B. bassiana* isolates, using PCR amplification with *B. bassiana*-specific primer and nucleotide sequence analysis of ITS region, confirmed only three isolates as *B. bassiana* (now named as BbR1, BbR2, and BbR3) along with MTCC 2028 reference isolate. Comparative RAPD-PCR amplification with ten different RAPD primers showed that all the three isolates were closely related. Genetic relatedness dendrogram developed, using RAPD-PCR data by UPGMA, measured the quantitative description of genetic diversity and showed that the standard isolate MTCC2028 was 58% similar to new isolates. *B. bassiana* isolates achieved their maximum growth during the optimum incubation period of 7 days. Significant mortality rates of the tobacco caterpillar, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae), were recorded at all the tested concentrations of conidial suspensions (10^3 to 10^7 conidia ml^{-1}) of *B. bassiana*. BbR2 was found to be the most potential local isolate, causing the maximum mortality (83.33%) of *S. litura*, with LC_{50} value of 935.663 conidia ml^{-1}, followed by BbR3 (74.33%), BbR1 (72.22%), and BbM1 (67.12%) isolates.

Keywords: *Beauveria bassiana*, Isolates, Molecular identification, *Spodoptera litura*, Efficacy

Background

Insect pests continue to pose a major threat for achieving higher production of agricultural crops. India loses 16.80% of its crops due to various insect pests, resulting in huge annual revenue loss estimated to be 36 billion USD (Dhaliwal et al. 2015). Synthetic pesticides, as an integral part of modern crop management practices, are significantly contributing to the improved agricultural production in the country by minimizing yield losses. To manage the insect pests, consistent indiscriminate applications of synthetic chemicals have resulted in serious negative concerns. These include emergence of high levels of pesticide resistance in many pest species, environmental toxicity, fishery losses, ground and surface water contamination, depletion of rhizosphere microflora, food safety hazards, and human health concerns (Shetty and Sabitha 2009).

Tobacco caterpillar, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae), is an extremely serious pest of agricultural crops. It causes severe damage to more than 115 plant species including pulses, cotton, cabbage, cauliflower, castor, groundnut, and oilseed crops (Atwal and Dhaliwal 2009). The pest is known to cause severe damage to non *Bt* cotton, cabbage, cauliflower, sunflower, etc. in Punjab. The exhaustive use of insecticides for its management has led to the problem of resistance. Growing public concern over potential health hazards of synthetic pesticides has changed the research towards more environmental friendly insect pest management tactics.

Among biocontrol agents, a number of entomopathogenic fungal species (EPF) have shown a promising

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potential against number of insect pests. *Beauveria bassiana* (Bals.) Vuill. is one of the most common EPF, occurring in multiple hosts, and is ubiquitous in most regions of the world (Meyling et al. 2009 and Roy et al. 2010). The genes involved in virulence of *B. bassiana* have been compiled by Valero-Jimenez et al. (2016).

Polymerase chain reaction (PCR)-based random amplified polymorphic DNA (RAPD) has been extensively used to differentiate strains of *B. bassiana*. RAPD analysis has been utilized to generate unique PCR products or amplicons in filamentous fungal species of interest to be converted into species-specific sequence-characterized amplified region (SCAR) markers (Li et al. 1999). Besides these, nucleotide differences in the ITS regions of the ribosomal DNA (rDNA-ITS) have been used to assess the genetic variability among *Beauveria* spp. (Imoulan et al. 2017 and Badotti et al. 2017). Hegedus and Khachatourians (1996) developed a *B. bassiana*-specific PCR-based technology for molecular identification of *B. bassiana* isolates.

B. bassiana has long been recognized as a potential biocontrol agent, and the compatibility of this fungus with chemical insecticides for use in IPM has already been evaluated and established (Oliveira et al. 2003). Specific strains of *B. bassiana* have been developed into number of commercial biopesticides (BotaniGard®ES, BotaniGard®22WP, Naturalis®TNO and Mycotrol®) for control of various insect pests on wide range of crops, vegetables, and forest trees (Strasser et al. 2001). In India, the commercial biopesticides have been recommended for control of different insect pests on different crops, but their application has shown only a reduced efficiency attributed to differences in susceptibility of target insect pests or non-adaptability to Indian agro-climatic conditions. Therefore, there is a need to isolate region-specific biopesticide strains to enhance the efficacy of such biopesticides for pest management.

This study aimed to isolate and characterize new isolates of the entomopathogenic fungus, *Beauveria* species from field soils of Punjab, India, and to evaluate their virulence against *S. litura*, one of the economic pest in the region.

Materials and methods

Beauveria bassiana isolates

Standard culture of *B. bassiana* (BbM1) MTCC 2028 was procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India (Varma et al. 1988). In addition, *Beauveria*-related species were isolated from different field soils of the state of Punjab, India, using *Beauveria*-specific selective media (Shimazu and Sato, 1996).

A total of 48 soil samples were collected from different districts of Punjab from fields of different crops (cotton, rice, vegetables, and pulses). About 100 g soil was collected from four different locations up to the depth of 5 cm of 1-acre field. The soil samples from different parts of a field were pooled, and a representative sample of 50 g was suspended in 500 ml of sterile distilled water in a conical flask. The contents were kept on a rotary shaker (200 rpm, 30 min, RT) to dislodge microbial cells and fungal spores from soil particles. After allowing the soil particles to settle for 15 min, 100 μl of a 10^{-2} dilution of the supernatant was plated on *Beauveria*-specific medium in Petri plates (90 mm). The plates were incubated at 25 °C in a BOD incubator for 7 days. The optimal conditions and optimal media for the isolates were followed as per Dhar et al. (2016).

Morphological characterization

After 7 days of incubation, morphological characteristics of fungal colonies were recorded for their identification as *B. bassiana* (Draganova et al. 2010). Bits of mycelial growth with sporulating mass were suspended in 1.0 ml of sterile water by vortexing, and 50 μl of the same was surface plated on BS medium in fresh Petri plates (90 mm). The plates were incubated at 25 °C in a BOD incubator to allow growth of fungal colonies from single spores. After 4 days of incubation, individual colonies were transferred on slants of BS medium and master cultures were allowed to grow and then stored at 4 °C until used.

Infectivity of *B. bassiana* isolates against *Galleria mellonella* larvae

The entomopathogenic potential of 13 isolated fungal isolates (out of 48) showing *B. bassiana* characteristics was tested against *G. mellonella* larvae, following the method of Ali-Shtayeh et al. (2002). Isolates were grown on PDA (potato dextrose agar) medium in slants for 10 days until sporulation. Small fungal hyphae bits were incised into 1.5-ml micro centrifuge tubes containing 1 ml of sterilized distilled water and 0.05% Triton X. The tubes were then vortex mixed. Ten third-instar *G. mellonella* larvae (obtained from infected spent honey combs from Apiculture Farm, Punjab Agricultural University, Ludhiana, India) were dipped into each fungal suspension for 5 s. The larvae were then placed on moisturized sterile filter papers in sterile Petri plates and incubated in the dark at 25 °C. Petri dishes were checked daily for larval mortality (Fig. 1). The control larvae were dipped in Triton X solution and incubated. After inoculating the *G. mellonella* larvae with *Beauveria* isolate, insect mortality was observed after 7 days with fungal mycelium emerging out of the larvae. Small bits of fungal growth emerging out of dead larvae were inoculated onto PDA agar until sporulation was observed. Based on infectivity and morphological features, three isolates were selected for further studies.

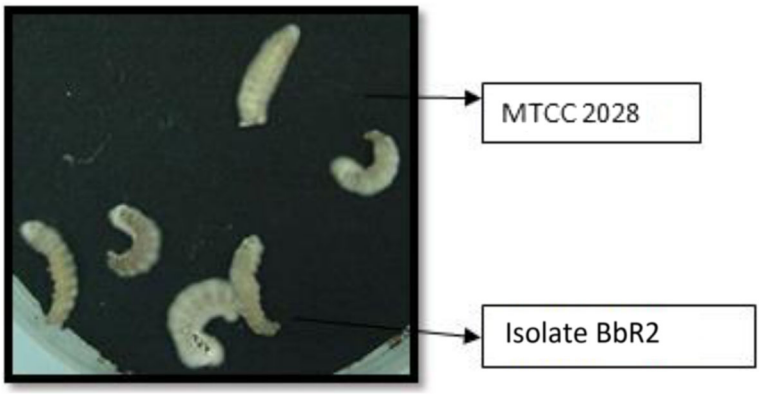

Fig. 1 *Galleria mellonella* larvae showing infection with two different fungal isolates establishing their entomopathogenic characteristics. *Galleria mellonella* larvae infected with fungal isolate BbR2 and *Beauveria bassiana* MTCC 2028

Molecular analysis of *B. bassiana* isolates for genetic relatedness

DNA extraction

Beauveria isolates were cultivated in (50 ml) PD broth at 25 °C. The mycelial biomass was harvested on a filter paper and washed three times with sterile distilled water. Fresh mycelial biomass (100 mg) for each isolate was grounded to fine powder in liquid nitrogen, using a sterilized pestle and mortar. The biomass powder, suspended in CTAB solution (5 ml), was incubated at 65 °C for 30 min. The solution was extracted by chloroform:isoamyl alcohol (24:1) solution (5 ml) by vortexing. After centrifugation (10,000 rpm, 10 min), the upper DNA containing aqueous phase was transferred into a fresh centrifuge tube (15 ml) and total DNA was precipitated by an equal volume of isopropanol (5 ml). The DNA was collected in a pellet by centrifugation (12,000 rpm, 5 min); the pellet was washed once with 70% ethanol, allowed to air dry for 5–10 min, and was suspended in 100 μl of TE (10 mM Tris.cl-1 mM EDTA, pH 8.0) solution.

Molecular identification

Total DNA from different fungal isolates was PCR amplified, using *B. bassiana*-specific primers (P1–5′-AAGC TTCGACATGGTCTG-3′ and P3–5′-GGAGGTGGTGA GGTTCTGTT-3′ primers) (Hegedus and Khachatoriuans 1996), using a PCR amplification program (95 °C for 5 min (preheating), 95 °C for 1 min, 54 °C for 1 min, 72 °C for 2 min (28 cycles), 72 °C for 10 min; final extension) and stored at 4 °C until used. The amplification of the desired DNA band of 524 bp was observed on 1.2% agarose gel in TAE. ITS region from all the three isolates was amplified, using bbITS-F/SR6R (aagtataagtcgtaacaagg) and bbITS-R/LR1 (ggttggtttctttcct) (Lane et al. 1985 and Vilgalys and Hester 1990) and cloned in pGEM-Teasy vector. The nucleotide sequence was determined and compared to NCBI database for confirmation of these isolates as *B. bassiana.*

Analysis of RAPD-PCR profiles for genetic relatedness

The genetic relatedness among different *B. bassiana* isolates along with standard *B. bassiana* MTCC 2028 was assessed through comparative RAPD-PCR (random amplified polymorphic DNA-polymerase chain reaction) employing RAPD primers (OPF-12, OPF-03, OPE-01, OPB-07, OPB-10, OPC-04, OPG-03, OPL-08, OPN-10, and OPP-04), using an annealing temperature of 42 °C (38 cycles). The amplified DNA fragments were analyzed through agarose gel electrophoresis and banding pattern photographed in gel documentation system. RAPD banding patterns were manually scored and processed by NTSYS software for developing genetic relatedness dendrogram by UPGMA (NTSYS pc version 2.02) (Rholf 1998), a measure of quantitative description of genetic diversity. All the individual bands in RAPD banding profile of different *B. bassiana* isolates were scored for presence (1) or absence (0) of all the individual bands.

Spodoptera litura bioassay

S. litura larvae were collected from infested castor plants from Punjab Agricultural University, Ludhiana, and maintained in the laboratory at 25 °C and 70–75% (RH). Subsequently, the larvae were reared individually on castor leaves. Castor leaves were surface sterilized by alcohol to prevent any possible contamination (Sasidharan and Varma 2005).

The conidia of the three isolates and the standard were revived on PDA medium plates at 25 ± 2 °C until they developed dense conidiation (7–10 days). The conidia were harvested from 2-week-old cultures by flooding the plates with sterile aqueous (0.05%) Tween-80 (Sigma USA) solution. The suspension was filtered through sterile muslin cloth to remove any mycelial fragments (Sasidharan and Varma 2005). The concentration of conidia was determined by a Neubauer hemocytometer, under a phase contrast microscope (Zeiss), and adjusted by dilution with Tween-80 solution to 1.0×10^8 conidia/

ml, which served as the stock suspension. The stock concentration was diluted to different suspensions with conidial count (1×10^3, 1×10^4, 1×10^5, 1×10^6, and 1×10^7 conidia ml^{-1}) for each *B. bassiana* isolate.

The second instar of *S. litura* larvae was dipped in different concentrations of conidial suspensions of each *B. bassiana* isolate for 5 s in a plastic container (8 cm diameter and 10 cm height). After dipping, the larvae were shifted on a sterile filter paper for soaking excess moisture and then released on a surface-sterilized castor leaves in individual sterile containers. The food was changed daily until larval death or pupation. In the control, the larvae were dipped in 0.05% Tween-80 solution. Daily observations were made to observe the development of fungal mycosis and consequent insect mortality.

Statistical analysis

The corrected mortality rate was calculated, using Abbott's formula (Abbott 1925). The median lethal concentrations (LC_{50}) were also estimated by probit analysis, using the computer Program POLO (LeOra Software, 1987). The data were subjected to ANOVA, and the least significant difference was calculated, using a factorial CRD design.

Results and discussion

B. bassiana isolates from soil

Out of 48 soil samples collected, 13 fungal species on selective BS medium showed morphological and cultural characteristics similar to *Beauveria* species. Colonies were round, lightly raised with white powdery surface, lightly downy with circular rings. White powdery translucent mycelium shows radial growth. Condiospores were densely clustered in whorls, one celled, hyaline, smooth, and short.

Screening of *Beauveria* isolates by *Galleria* infectivity method

Virulence of all the 13 fungal isolates was evaluated first against *G. mellonella* larvae (Ali-Shtayeh et al. 2002). Out of 13 fungal isolates, only the 3 isolates 2, 9, 31 (now named as BbR1, BbR2, and BbR3) effectively caused mortality of *G. mellonella* larvae within 5–7 days coupled with a white muscardine growth of fungal mycelium (typical of *B. bassiana*) (Fig. 1).

In order to acquire cultures with optimum virulence, the selected 3 fungal isolates and the standard *B. bassiana* (MTCC 2028 culture, called as BbM1) were re-isolated from dead *G. mellonella* larvae that had been infected with each respective fungal isolate. Small bits of the fungal mass were transferred and cultured in PDA medium slants. All the cultures, processed for a single spore isolation and cultured from an individual isolated fungal colony, were used as master cultures for all subsequent studies.

Molecular identification of *B. bassiana* isolates

Total DNA, isolated from all the 13 different fungal isolates, was PCR amplified by *B. bassiana*-specific primers P1 and P3. DNA fragment of size approximately 520 bp was amplified. The PCR product of only the same 3 isolates, viz. BbR1, BbR2, and BbR3, and BbM1 reference MTCC2028 isolate was close to the expected 524 bp. This indicated that out of the 13 fungal isolates, only 3 strains (Fig. 2) were *B. bassiana*. The rest showed different types of amplification patterns, which could be non-virulent species of *Beauveria* that are closely related to *B. bassiana* in morphological characteristics. Based on ITS nucleotide sequence analysis, the same 3 isolates were shown to have maximum homology with *B. bassiana* sequences in the NCBI database. The nucleotide sequences of the 3 isolates (BbR1, BbR2, and BbR3) were submitted to NCBI GenBank with accession nos. MG670098.1, MG670100.1, and MG670102.1, respectively (Geetika Banta, Personal Communication).

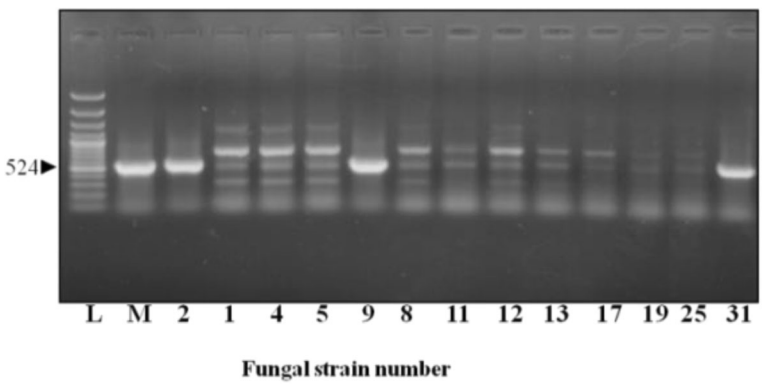

Fig. 2 PCR amplification with *Beauveria bassiana*-specific primers from different fungal isolates. M is the reference isolate *B. bassiana* MTCC 2028; L is 100-bp DNA ladder (Fermentas Life Sciences)

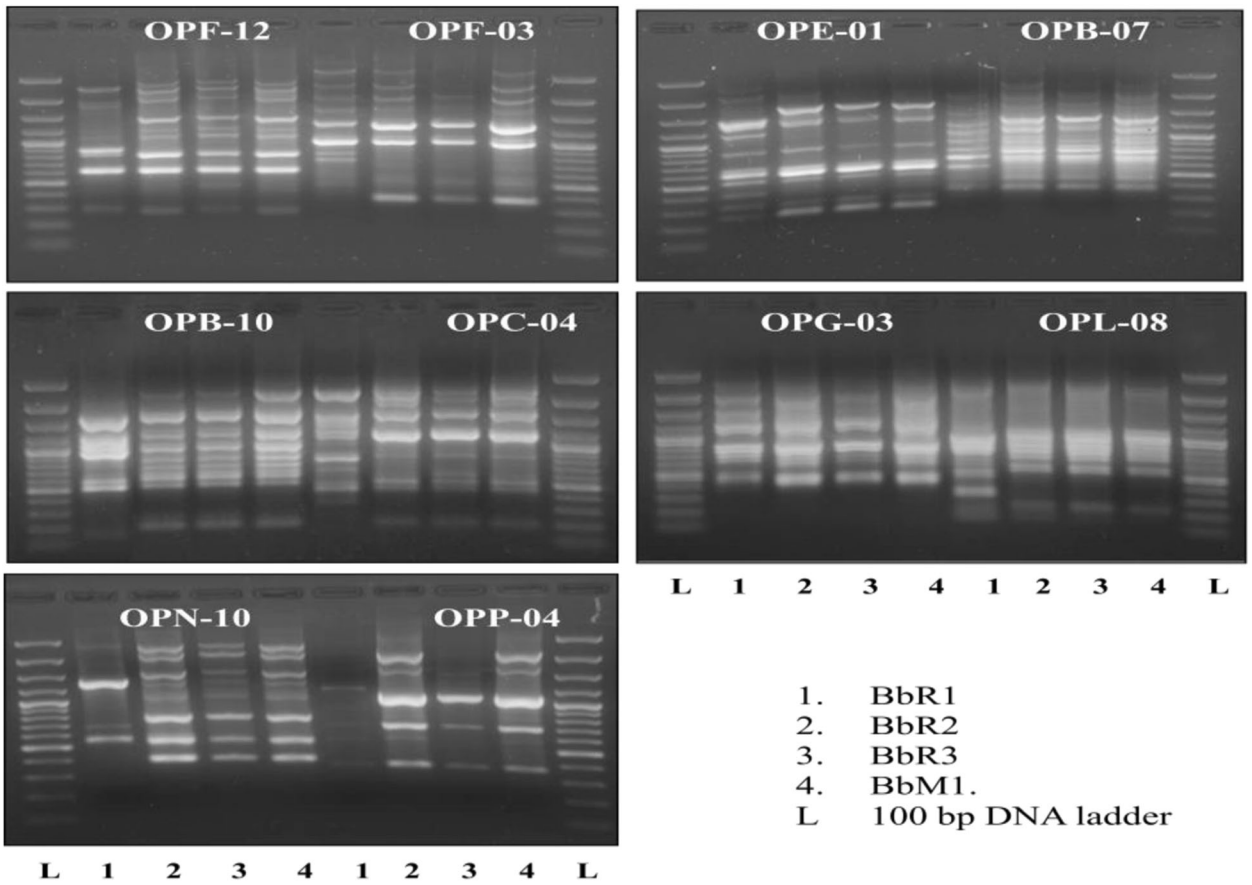

Fig. 3 Comparative RAPD-PCR amplification profile (banding pattern) of different *Beauveria bassiana* isolates with ten different RAPD primers

Genetic relationship of *B. bassiana* isolates

Comparative RAPD-PCR was used to examine genetic differences among the new *B. bassiana* isolates, including *B. bassiana* MTCC 2028. The comparative RAPD-PCR banding profile was obtained by ten different random primers (Fig. 3). The comparative banding pattern identified polymorphic marker bands suggesting molecular differences among these *B. bassiana* isolates, including the reference MTCC2028 isolate.

Genetic relatedness dendrogram as developed, using RAPD data by UPGMA, was used to measure the quantitative description of these genetic differences. This dendrogram indicated that BbR1, BbR2, and BbR3 were closely related. The maximum of 94% similarity was observed between isolate BbR1 and BbR3, which was similar to BbR2 with 88% similarity (Fig. 4).

However, these new isolates showed 58% similarity with BbM1. High genetic divergence of BbM1 from the new

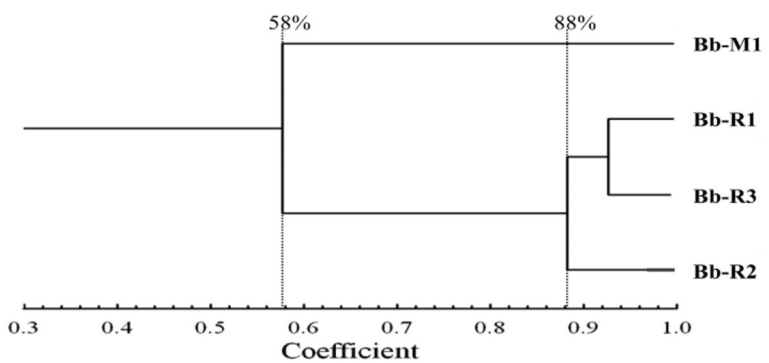

Fig. 4 RAPD-PCR data-based genetic relatedness dendrogram among different *Beauveria bassiana* isolates

isolates from Punjab soils in this study may be due to the reason that the former was originally isolated in 1988 from IISR, Lucknow, a distantly located geographic region of India (Varma et al. 1988), and faced repeated sub-culturing at MTCC, Institute of Microbial Technology, Chandigarh.

Various workers have effectively used the RAPD to assess the genetic diversity of different isolates of *B. bassiana* (Kaur and Padmaja 2008; Imoulan et al. 2016; Prabhukarthikeyan et al. 2017). The studies reported the high genetic diversity among local isolates of *B. bassiana*, which supported the diversity of maximum 42% between standard MTCC2028 and the obtained new isolates.

Efficacy against *Spodoptera litura*

All *B. bassiana* isolates were infectious but differed in their aggressiveness, i.e., pest mortality. No larval mortality was observed up to 48 h post infection, irrespective of *B. bassiana* isolate. However, after 72 h, mortality was observed in the case of all the isolates and conidial concentrations, ranged between 2.33 and 39.81% (Fig. 6). The mortality rate increased with the increasing number of conidial concentration used. BbR2 caused the maximum 39.81% mortality, followed by BbR1 (38.14%), BbR3 (32.66%), and BbM1 (32.22%). Isolate BbR2 caused the maximum mortality at the conidiospore concentration each of 10^6 and 10^7 conidia ml^{-1} after 96, 120, and 144 h of treatment and was significantly superior to all isolates (Fig. 6).

After 168 h of infection, *B. bassiana* isolate BbR2 was found to be the most effective one, causing a maximum mean mortality of 83.33%, followed by BbR3 (74.33%) and BbR1 (72.22%) at the conc. of 10^7 conidia ml^{-1}. Standard isolate BbM1 caused the least mortality (67.12%) at the conc. of 10^7 conidia ml^{-1} after 168 h of treatment (Figs. 5 and 6).

Comparison of LC_{50} values (overlap of confidence intervals) provided a significant difference among the isolates. The most virulent isolate BbR2 (935.663 conidia ml^{-1}) had the lowest LC_{50} values, while BbR1 (4735.68), BbR3 (5725.68), and BbM1 (7934.7) had comparatively high LC_{50} values (Table 1). Thus, the isolate BbR2, at the concentrations of 10^6 and 10^7 conidia ml^{-1}, was significantly superior over other ones of *B. bassiana* after all the different hours of infection. Monitoring of infected larvae, which survived mortality after 168 h, suggested that some of these larvae pupated but they did not emerge as adults. Isolate BbR2 proved to be the best among the four isolates.

A significant decrease in the larval period was observed due to *B. bassiana* infection as compared to the control. These results corroborate the findings of Batta and Abu-Safieh (2005) who reported a decrease in the life cycle of red flour beetle, *Tribolium castaneum* (Herbst), when treated with *Metarhizium anisopliae*. Khachatourians (1986) also suggested that EPF caused the death of their host due to exhaustion of nutrients and liberation of toxins in the hemolymph. So, nutritional deficiency and toxins produced by EPF drastically affect the development of an insect. Earlier, Sharma et al. (1994) reported physiological changes in *Heliothis armigera* larvae after injecting them with filtrate of *B. bassiana* culture and found that the toxins destroy the normal balance of physiological system. In the present study, apart from causing insect mortality, *B. bassiana* isolates caused pupal and adult deformities in *S. litura.*

Moorthi et al. (2015) tested the efficacy of isolates collected from southern India against *S. litura* and found the LC_{50} value of 1.3×10^3, which was comparatively higher than BbR2 isolate (935.663). Ortiz et al. (2016) reported LC_{50} of 1.07×10^7 to 1.31×10^{10} condia ml^{-1} of different

Fig. 5 *Beauveria bassiana* infection on *Spodoptera litura* larvae on the seventh day. White fluffy fungal mycelium is seen covering whole of the insect

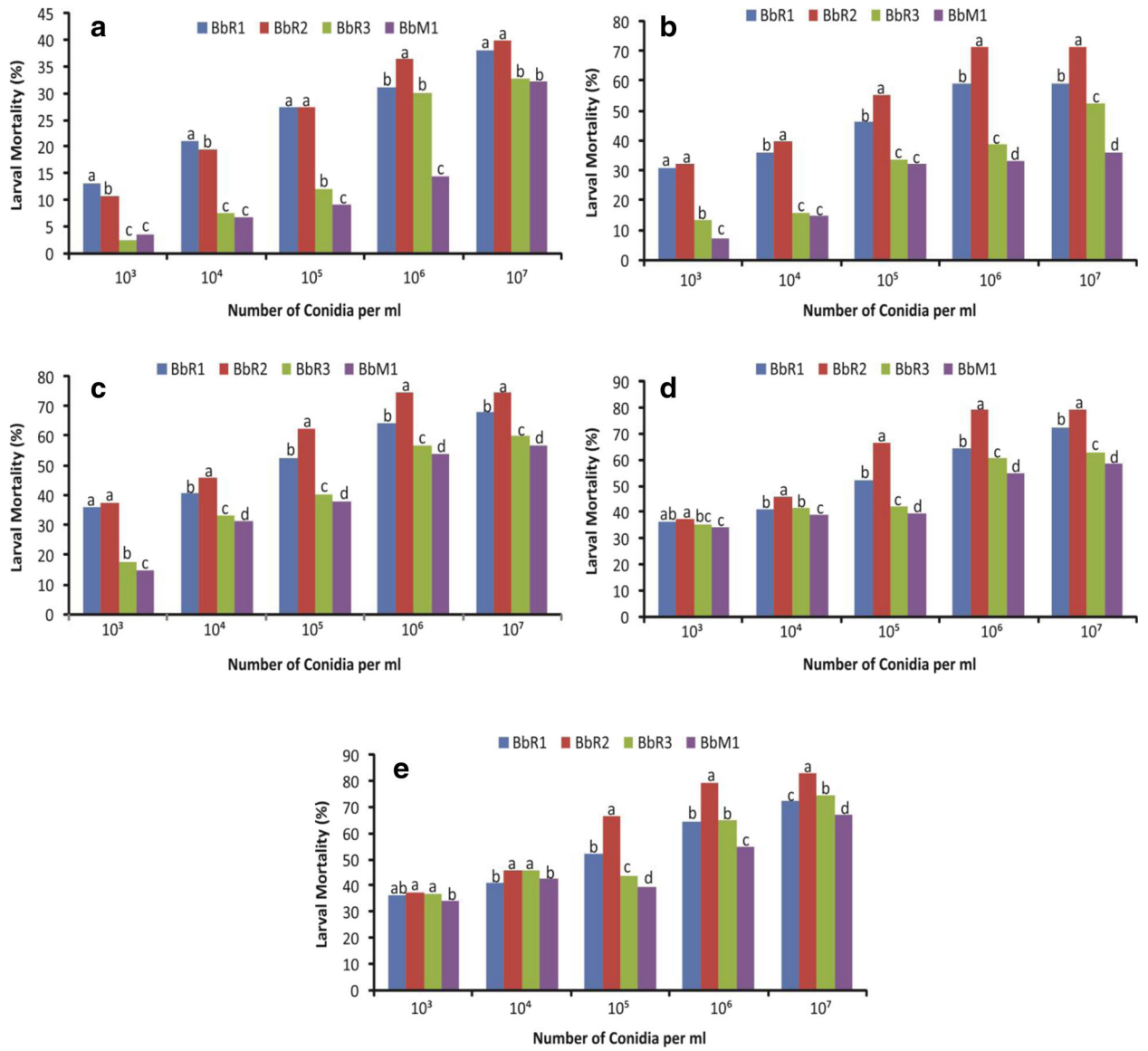

Fig. 6 Mortality of *Spodoptera litura* larvae by different *Beauveria bassiana* isolates after different intervals post infection. **a** 72 h. **b** 96 h. **c** 120 h. **d** 144 h. **e** 168 h. Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$)

Table 1 LC_{50} estimates of different *Beauveria bassiana* isolates from bioassays against second-instar larvae of *Spodoptera litura*

Isolates	*n*	Heterogeneity	LC_{50}	Slope ± SE	X^2 (df)	Limits
BbR1	30	0.07	4735.680	0.214 ± 0.076	0.1968 (3)	2.62 to 45,428.899
BbR2	30	0.13	935.663	0.344 ± 0.086	0.3805 (3)	12.633 to 5794.919
BbR3	30	0.12	5725.680	0.214 ± 0.086	0.3904 (3)	11.625 to 65,197
BbM1	30	0.15	7934.700	0.173 ± 0.074	0.4552 (3)	6.9569 to 77,197

n no. of insects treated, *df* degrees of freedom, LC_{50} lethal concentration at which 50% of the insect population gets killed

isolates of *B. bassiana* (Bb-Hy, Bb-Rhy, and Bb-13) against soybean weevil *Rhyssomatus nigerrimus.* An Indian isolate DOR *B. bassiana* (developed by ICAR-Directorate of Oilseed Research, Hyderabad) was found effective for management of *Helicoverpa armigera* in sunflower (Kumar and Kaur 2017). Nazir et al. (2019) tested isolates of *B. bassiana* (BB-72 and BB-252) against green peach aphid, *Myzus persicae*, where they recorded high mortality of aphids (95 and 91%, respectively) after 10 days of treatment. Different isolates from different countries have different potential in the management of different insect pests. Therefore, the isolates of *B. bassiana* found in this study may hold a significance in the management of insect pests in Punjab conditions.

Conclusions

Three new isolates of *B. bassiana* were isolated from the soils of Punjab, India. RAPD analysis resulted in 88% similarity among the isolates; however, these were 58% similar to the standard MTCC2028. A wide range of variations was recorded in the degree of virulence among the three isolates tested. *B. bassiana* isolate BbR2 showed a higher degree of virulence in the bioassay experiment than the other isolates, and the maximum mortality rate was recorded on the seventh day after treatment.

Field experimentation is necessary to substantiate the present findings on the relative efficacy of *B. bassiana* isolates against *S. litura*. Further laboratory experiments with different fungal isolates in relation to dosage, time, storage, and stability should be undertaken to find the optimum concentration and efficient application of fungal materials.

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Author's contributions

SD carried out the isolation, molecular and bioassay studies, and maintained the culture. VJ designed the bioassay experiment and did the statistical analysis of the bioassay data. VJ is also involved in preparation, reading, and approval of the manuscript. MJ has contributed equally in the molecular experiments. VKG conceptualized and designed the studies and read and approved the final manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not Applicable

Competing interests

The authors declare that they have no competing interests.

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