Metabolome heterogeneity in the isolates of entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin

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**ABSTRACT:** Entomopathogenic fungi are known to produce a multitude of low molecular weight secondary metabolites involved in different biological processes including fungal development, intercellular communication and interaction with other organisms in complex niches. In the present investigation, heterogeneity in metabolome profile of three isolates of *Beauveria bassiana* viz., MH590235 (TM), MK918495 (BR) and KX263275 (BbI8) were analyzed through GC-MS. Distinct differences in metabolite profile of the isolates were observed. A total of 63 metabolites were detected from all the isolates combined. Metabolites, 5-Oxotetrahydrofuran-2-carboxylic acid and undecane were found to be specific to BR isolate. Macrocyclic gamma lactones were detected in culture filtrates of BR and BbI8, oleic acid and hexadecanoic acid in TM and BR. An insecticidal compound, levoglucosan was detected in all the fungal isolates. Among the isolates, TM revealed higher variability in the metabolite production through PCA analysis. The metabolome of TM isolate contained compounds having several biological functions, viz., insecticidal and antimicrobial activity, lipid and fatty acid metabolisms and virulence enhancing factors.

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**INTRODUCTION**

Entomopathogens are considered as a promising component of Integrated Pest Management Programmes (Butt, 2001) among which fungal Biocontrol Agents (BCAs) are widely exploited in view of their broad spectrum activity and amenability for mass production. All BCAs are known for the production of enzymes and secondary metabolites responsible for pathogenicity. The cuticle degrading enzymes, viz., lipases, proteases and chitinases were targets of study from the time of discovery of mode of action of these fungal BCAs but descriptive studies on the secondary metabolite production by these agents are meagre.

Most often, the fungal BCAs secrete metabolites in extremely small quantities even under optimal conditions (Vey et al., 2001). Destruxins produced by *Metarhizium* spp. (Wahlman and Davidson, 1993), beauvericin and bassianolide by *Beauveria bassiana* (Xu et al., 2008; Xu et al., 2009), hirsutellin by *Hirsutella thompsonii* (Mazet and Vey, 1995) are the few metabolites widely studied. Little is known about the complete range of metabolites produced by most of the EPF. Though these fungi produce a wide array of bioactive compounds, the knowledge on specific role of a particular compound is lacking. Production of these metabolites may vary between genus, species and growth conditions (Kershaw et al., 1999; Amiri-Besheli et al., 2000; Wang et al., 2004).

Many studies have been conducted on virulence of several strains of *Beauveria* spp. on insect hosts, in particular, *B. bassiana* (Talaei-Hassanloui et al., 2006; Valero-Jiménez et al., 2014). Few studies demonstrated variation in host range of fungus within species and between species of *Beauveria* (Rohrlich et al., 2018). However, limited studies were carried out on the variation in metabolite profile among isolates of a particular species of fungal BCAs and hence the present study was undertaken to characterize variation in metabolite production among three isolates of *B. bassiana* grown under similar conditions.

**MATERIALS AND METHODS**

**Cultures and growth conditions**

*Beauveria bassiana* isolates bearing NCBI accessions MH590235, MK918495 and KX263275 were obtained from Department of Agricultural Entomology and Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India. Pure cultures of the isolates were
maintained at 28±5°C on Potato Dextrose Agar (PDA) medium for carrying out the study. Mycelial discs were cut from heavily sporulated culture plates using cork borer and inoculated into Potato Dextrose Broth (PDB) for extraction of metabolites.

**Extraction of secondary metabolites**

Isolates of *B. bassiana* were cultured in PDB for seven days after which culture filtrates were collected and adjusted to pH 2.0 with 37% (wt/vol) HCl. Metabolites were thrice extracted with an equal volume of ethyl acetate and the pooled ethyl acetate extracts of three biological replicates were dried using a rotary evaporator and re-suspended in HPLC grade methanol (1 ml). The extracts were then dried over Na₂SO₄ and evaporated under vacuum at 60º C to concentrate the metabolites. The metabolites were finally dissolved in HPLC grade methanol and utilized for GC-MS analysis (Strasser et al., 2000).

**Gas Chromatography- Mass Spectrometry (GC-MS)**

The samples were analyzed using a model Clarus SQ 8C (Perkin Elmer) equipped with a MSD detector (Perkin Elmer). The GC injector port temperature was set to 220°C, interface temperature at 250°C and source temperature was set at 220°C. The MS range was set to scan from 50 to 550 Da. The oven temperature was programmed to 75°C (hold 2 min), then to 150°C (10°C/min), then to 250°C (10°C/min). The injection volume of 1.0 μl and split ratio of 1:12 and the injector used was split less mode. Helium was used as the carrier gas in constant-flow mode of 1.0 ml/min. The DB-5 MS capillary standard non-polar column (Agilent Co., USA) with dimensions were 0.25mm OD x 0.25μm ID x 30 m length was used for analysis. The MS source was maintained at 220°C, 4.5e4 motor vacuum pressure and ionization energy was set to -70eV. The MS have inbuilt pre-filter which reduced the neutral particles. Interpretation of mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST14). The spectrum of the unknown component was compared with the spectrum of the known components stored in the inbuilt library.

Identification of the metabolites were performed using spectra of individual components transferred to the NIST mass spectral search programs MS Search 2.2v where they were matched against the NIST MS library. Biological function of these compounds was identified by mapping all the metabolites in the KEGG database and Metaboanalyst 2.0.

**Statistical analysis**

Principal Component Analysis (PCA) and heatmap construction combined with hierarchical clustering were performed using JMP software (version 14) using the data from GC-MS. Percentage area values were used as independent variables in this multivariate analysis. Metabolites were clustered using R software for heat map generation.

**RESULTS AND DISCUSSION**

Culture filtrates of three isolates of *B. bassiana* were extracted using ethyl acetate and the variability in metabolite profile of different isolates of *Beauveria bassiana* were assessed using GC-MS (Fig. 1, 2, 3). In the present investigation, inraspecific variation was observed in the metabolites extracted from culture filtrates of the three isolates of *B. bassiana*. 29 metabolites including alkanes, carboxylic acid derivatives, gluco pyranose and galactofuranose derivatives, unsaturated fatty acids, hexadecanoic acid derivatives were identified in TM isolate (Table 1, Fig. 1). 29 and 26 metabolites were detected in BR and BbI8 isolates mass spectrum respectively (Table 2, 3). Hyun et al. (2013) reported the presence of alcohols, amino acids, organic acids, phosphoric acids, purine nucleosides and bases, sugars, saturated fatty acids, unsaturated fatty acids, or fatty amides in 70 % methanol and 100 % hexane extracts of fruiting bodies of *Cordyceps bassiana*.

PCA is a powerful tool to selectively identify the major controlling factors contributing to differences between samples. It is hence applied in the present study for the comparative visualization and interpretations of the changes in the metabolites profiles of three *B. bassiana* isolates (Ramadan et al., 2006).

PCA biplot for ethyl acetate extracts of three isolates of *B. bassiana* are presented in Figure 4. In the biplot, PCA 1 explained 52% of the variation and PCA 2 explained 33.2% of the variation. Results showed clear distinction of TM from other isolates. TM was separated alone in PC 1 while BR and BbI8 were separated from TM along PC 2. Higher levels of palmitic acid and oleic acid were obtained in TM compared to BR.

The present investigation showed distinct differences in metabolite profile of *B. bassiana* isolates (Fig. 5, 6). BR and BbI8 isolates showed similarities in the level of metabolite production (Fig. 4). An anhydrase, 1,6-anhydro-α-D-Glucopyranose (levoglucosan) was detected in all the three isolates. A gamma lactone, 5-Oxotetrahydrofuran-2-carboxylic acid was found to be present in the isolates, TM and BbI8. Syed et al. (2018) reported the insecticidal activity of levoglucosan obtained through pyrolysis of bio-oils against cutworm larvae.
Metabolome heterogeneity in the isolates of *Beauveria bassiana*

The furan metabolite, 5-Oxotetrahydrofuran-2-carboxylic acid is a derivative of bassialone, an antimicrobial secondary metabolite produced by *B. bassiana* was detected in TM isolate in the present study. However, this was absent in BR isolate which showed clear variation in metabolite profile and this may indicate reduced virulence. 2-Deoxy-2-fluoro-1,6-anhydro-α-d-glucopyranose, 3-Hydroxy-2,3-dihydromaltol, 5-Hydroxymethylfurfural, Trioxsalen, Sucrose, Octadecanoic acid and 9,12-Octadecadienoic acid (Z,Z)- were detected in all the three isolates (Table 4) but the level of production varied among the isolates in terms of per cent area. This was confirmed through correlation analysis where positive significant correlation was detected between BR and Bb18 isolates of *B. bassiana* (Table 5, Fig. 7).

The metabolome of isolate TM was completely different from the other two isolates thus revealing least similarity with the other isolates (Fig. 5). Many studies were conducted in relation to the heterogeneity of secretome of entomopathogenic fungi under different growth conditions as well as extraction methods (Smedsgaard, 1997; Hyun et al., 2013; Oh et al., 2014). de Bekker et al. (2013) studied variation in metabolite production of *Metarhizium* and *Beauveria* during infectious and saprophytic growth.

Toxicity of secondary metabolites of *B. brongniartii* against pine caterpillar, *Dendrolimus tabulaeformis* was reported by Fan et al. (2008). Secondary metabolites of *B. brongniartii* was found to disable the immune mechanisms of *D. tabulaeformis*, and kill its host (Fan et al., 2013). In the present study, the metabolome of isolate TM was completely different from the other two isolates thus revealing least similarity with the other isolates (Fig. 5). In a previous study, isolate TM registered lowest values of LC50 (2.4 x 107 conidia ml-1) and LT50 (3.62 days) compared to the BR
Table 1. GC-MS based metabolite profile of *Beauveria bassiana* TM

| Sl. No | Compound Description | RT | Area (%) | Molecular weight (g/mol) | Molecular formula | Biological action | Reference |
|--------|----------------------|----|----------|--------------------------|------------------|-------------------|------------|
| 1      | Cyclohexanamine, N-3-butenyl-N-methyl- | 5.449 | 1.857 | 221.388 | C_{15}H_{27}N | Insecticidal, repellent, antimicrobial | Ibrahim *et al.*, 2001 |
| 2      | Undecane              | 5.674 | 0.348 | 156.31 | C_{11}H_{24} | Mild sex attractant of moths, alert signal for insects | Hölldobler and Wilson, 1990 |
| 3      | 2-Deoxy-2-fluoro-1,6-anhydro-á-d-glucopyranose | 6.275 | 0.761 | 182.15 | C_{6}H_{11}FO_{3} | Cell wall synthesis | Douglas, 2001 |
| 4      | 3-Hydroxy-2,3-dihydromaltol | 6.395 | 2.744 | 128.13 | C_{6}H_{8}O_{3} | - | - |
| 5      | 5-Oxotetrahydrofuran-2-carboxylic acid | 7.395 | 1.255 | 130.099 | C_{5}H_{6}O_{4} | Bassianolone derivative | Oller-Lopez *et al.*, 2005 |
| 6      | 5-Hydroxymethylfurfural | 7.500 | 3.083 | 126.11 | C_{6}H_{6}O_{3} | Fermentation inhibitor | Kadowaki *et al.*, 2018 |
| 7      | 1,3-Oxathiolane, 2-methyl-2-isopropyl- | 7.795 | 0.686 | 146.250 | C_{7}H_{14}OS | - | - |
| 8      | Cyclohexanone, 2-(2-butenyl)- | 8.766 | 0.364 | 150.221 | C_{10}H_{14}O | Antibacterial activity | Liu *et al.*, 2009 |
| 9      | Sulfurous acid, cyclohexylmethyl undecyl ester | 9.461 | 1.088 | 332.543 | C_{18}H_{36}O_{3}S | Insecticidal | Domon *et al.*, 2018 |
| 10     | 1,3-Propanediol, 2-methyl-2-propyl- | 9.646 | 0.827 | 132.203 | C_{7}H_{16}O_{2} | Lipid metabolism | Liu *et al.*, 2015 |
| 11     | Trioxsalen             | 9.991 | 0.836 | 228.24 | C_{14}H_{12}O_{3} | Antimicrobial | Gowri *et al.*, 2011 |
| 12     | Sucrose                | 10.832 | 8.207 | 342.297 | C_{12}H_{22}O_{11} | Source for growth and spore production | Samsinakova, 1966 |
| 13     | á-D-Glucopyranose, 1,6-anhydro- | 11.542 | 0.838 | 162.141 | C_{6}H_{10}O_{5} | Insecticidal | Syed *et al.*, 2018 |
| 14     | 1,6-Anhydro-á-d-galactofuranose | 13.663 | 4.054 | 162.141 | C_{6}H_{10}O_{5} | Cell wall component | Bernabe *et al.*, 2011 |
| 15     | 2-Imidazolidinethione  | 13.908 | 4.060 | 102.158 | C_{3}H_{6}N_{2}S | - | - |
| 16     | á-D-Glucopyranose, 4-O-á-D-galactopyranosyl- | 14.548 | 2.726 | 342.297 | C_{12}H_{22}O_{11} | Cell wall component | Bernabe *et al.*, 2011 |
| 17     | Palmitic acid          | 21.271 | 16.273 | 256.43 | C_{16}H_{32}O_{2} | Pesticidal activity, Lipid peroxidation | Vivekanadan *et al.*, 2018 |
| 18     | 9,12-Octadecadienoic acid (Z,Z)- | 24.447, 26.078 | 5.522 | 280.4 | C_{18}H_{32}O_{2} | Fatty acid metabolism | Zhang *et al.*, 2012 |
| 19     | 9-Octadecenoic acid, (E)- | 24.562 | 15.968 | 282.4614 | C_{18}H_{34}O_{2} | Fatty acid metabolism | Brennan *et al.*, 1975 |
| 20     | Octadecanoic acid      | 24.977 | 3.766 | 284.48 | C_{18}H_{36}O_{2} | Fatty acid metabolism | Zhang *et al.*, 2012 |
| 21     | Ethyl linoleate        | 25.332 | 0.379 | 308.4986 | C_{20}H_{36}O_{2} | Fatty acid metabolism | Zhang *et al.*, 2012 |
| 22     | Glycidyl palmitate     | 27.413 | 2.950 | 312.494 | C_{19}H_{36}O_{3} | Fatty acid metabolism | Zhang *et al.*, 2012 |
| 23     | Eicosanoic acid, ethyl ester | 28.909 | 0.363 | 340.592 | C_{22}H_{44}O_{2} | Antimicrobial activity | Suresh *et al.*, 2014 |
| 24     | Butyl linoleate        | 29.774 | 1.760 | 336.56 | C_{22}H_{40}O_{2} | Fatty acid metabolism | Zhang *et al.*, 2012 |
| 25     | Glycidyl oleate        | 29.854 | 2.540 | 338.532 | C_{21}H_{38}O_{3} | Fatty acid metabolism | Zhang *et al.*, 2012 |
| 26     | 1,3-Distearoylglycerol | 30.204 | 0.436 | 568.924 | C_{33}H_{68}O_{5} | Enhancement of virulence | Ortiz-Urquiza *et al.*, 2016 |
Table 2. GC-MS based metabolite profile of *Beauveria bassiana* BR

| Sl. No | Compound | RT (min) | Area (%) | Molecular weight (g/mol) | Molecular formula | Biological action | Reference |
|--------|----------|----------|----------|--------------------------|-------------------|------------------|-----------|
| 1      | 2-Deoxy-2-fluoro-1,6-anhydro-α-d-glucopyranose | 3.013 | 8.110 | 182.15 | C₆H₁₁FO₅ | Cell wall synthesis | Douglas, 2001 |
| 2      | Dihydrothiophenone | 3.574 | 0.729 | 102.151 | C₄H₆OS | Insecticidal, nematicidal | Champagne *et al.*, 1986; Hudson and Toers, 1991 |
| 3      | 2-t-Butyl-5-propyl-[1,3]dioxolan-4-one | 4.174 | 0.458 | 186.251 | C₁₀H₁₈O₃ | Fungitoxic | Horsefall and Lukens, 1965 |
| 4      | Thymine | 5.414 | 5.303 | 126.11 | C₅H₆N₂O₂ | Pyridine metabolism | Liu *et al.*, 2015 |
| 5      | Nonane, 2-methyl-5-propyl- | 5.664 | 0.559 | 184.367 | C₁₃H₂₈ | Insect growth regulator | Mian and Mulla, 1982 |
| 6      | 3-Hydroxy-2,3-dihydromaltol | 6.425 | 9.919 | 128.13 | C₆H₈O₃ | - | - |
| 7      | Cyclohexane, 1,1'-dodecylidenebis [4-methyl- | 7.040 | 0.431 | 362.6752 | C₂₆H₅₀ | Insecticidal, repellent, antimicrobial | Ibrahim *et al.*, 2001 |
| 8      | (S)-(−)-1-Amino-2-(methoxymethyl)-pyrrolidine | 7.365 | 2.820 | 130.19 | C₆H₁₄N₂O | Antimicrobial | Dumoulin *et al.*, 2010 |
| 9      | 5-Hydroxymethylfurfural | 7.500 | 3.083 | 126.11 | C₆H₆O₃ | Fermentation inhibitor | Kadowaki *et al.*, 2018 |
| 10     | Coumarin-6-carboxaldehyde | 7.770 | 1.326 | 174.155 | C₁₀H₆O₃ | Antimicrobial | Al-Majedy *et al.*, 2017 |
| 11     | 1-Decanamine | 7.980 | 0.673 | 269.517 | C₁₀H₂₀N | - | - |
| 12     | 1-(Methylthio)-3-pentanone | 8.331 | 1.330 | 132.23 | C₅H₁₀OS | - | - |
| 13     | N-Nitroso-2,4,4-trimethylazolidine | 8.766 | 0.831 | 144.172 | C₁₂H₁₂N₂O₂ | Antimicrobial, Anti-inflammatory | Kim *et al.*, 2001 |
| 14     | 2-Hydroxy-3-methylsucinic acid | 9.086 | 0.632 | 148.114 | C₅H₈O₄ | TCA cycle derivative | Hyun *et al.*, 2013 |
| 15     | 2,2-Dimethylcyclopropanecarboxylic acid | 9.466 | 2.422 | 114.14 | C₈H₁₀O₂ | - | - |
| 16     | Hydroxydocosahexaenoic acid | 9.666 | 1.114 | 344.5 | C₂₂H₃₃O₃ | Antibacterial | Mil-Homens *et al.*, 2012 |
| 17     | Trioxsalen | 9.986 | 2.949 | 228.24 | C₁₄H₂₀O₃ | Antimicrobial | Gowri *et al.*, 2011 |
| 18     | 1,2-Heptanediol | 10.161 | 0.851 | 132.2 | C₇H₁₅O₂ | - | - |
| 19     | Sucrose | 10.821 | 14.363 | 342.297 | C₁₂H₂₂O₁₁ | Source for growth and spore production | Samsinakova, 1966 |
Table 3. GC-MS based metabolite profile of *Beauveria bassiana* Bb18

| Sl. No | Compound                                                                 | RT  | Area (%) | Molecular weight (g/mol) | Molecular formula | Biological action                                                                 | Reference             |
|-------|---------------------------------------------------------------------------|-----|----------|--------------------------|-------------------|-----------------------------------------------------------------------------------|-----------------------|
| 1     | Undecane                                                                  | 4.249 | 1.086   | 184.37                   | C₁₃H₂₈             | Mild sex attractant of moths, alert signal for insects                            | Hölldobler and Wilson, 1990 |
| 2     | 2-Nonadecanone, 2,4-dinitrophenylhydrazine                                  | 4.334 | 0.552   | 462.635                  | C₂₅H₄₂N₄O₄         | -                                                                                | -                     |
| 3     | Clindamycin                                                               | 5.389 | 2.804   | 424.98                   | C₁₈H₃₃ClN₂O₅S      | Antibiotic                                                                        | Woappi et al., 2016   |
| 4     | 2-Deoxy-2-fluoro-1,6-anhydro-á-d-glucopyranose                            | 6.315 | 1.322   | 182.15                   | C₆H₁₀FO₅           | Cell wall synthesis                                                              | Douglas, 2001         |
| 5     | 3-Hydroxy-2,3-dihydromaltol                                               | 6.435 | 4.010   | 128.13                   | C₆H₁₂O₃            | -                                                                                | -                     |
| 6     | 5-Oxotetrahydrofuran-2-carboxylic acid                                    | 7.400 | 1.985   | 130.099                  | C₅H₆O₄            | Bassianolone derivative                                                          | Oller-Lopez et al., 2005 |
| 7     | 5-Hydroxymethylfurfural                                                  | 7.555 | 1.544   | 126.11                   | C₆H₁₀O₃            | Fermentation inhibitor                                                           | Kadowaki et al., 2018 |
| 8     | 1,2,3-Butanetriol                                                         | 8.391 | 0.951   | 106.121                  | C₆H₁₂O₃            | -                                                                                | -                     |
| 9     | 2-Methoxy-4-vinylphenol                                                   | 8.821 | 0.776   | 150.177                  | C₄H₈O₃            | -                                                                                | -                     |
| 10    | 3-Propylglutaric acid                                                     | 9.441 | 3.237   | 174.196                  | C₮₈H₱₄O₄           | -                                                                                | -                     |
| 11    | 1,3-Dioxane-5-methanol, 4,5-dimethyl-                                    | 9.676 | 1.094   | 146.186                  | C₅H₈O₃            | -                                                                                | -                     |
| 12    | Trioxsalen                                                                | 10.021 | 1.679  | 228.24                   | C₈H₂₆O₃            | Antimicrobial                                                                    | Gowri et al., 2011    |
| 13    | Sucrose                                                                   | 10.556 | 9.462   | 342.297                  | C₁₂H₂₂O₁₁          | Source for growth and spore production                                            | Samsinakova, 1966     |
| 14    | á-D-Glucopyranose, 1,6-anhydro-                                           | 11.532 | 0.568   | 162.141                  | C₈H₁₈O₃            | Insecticidal                                                                      | Syed et al., 2018     |
| 15    | Benzocycloheptano[2,3,4-]jisoquinoline, 4,5,6,6 tetrahydro-1,9-dihydroxy-2,10-dimethoxy-5-methyl- | 12.482 | 0.584   | 341.407                  | C₂₀H₂₃NO₄          | -                                                                                | -                     |
Metabolome heterogeneity in the isolates of *Beauveria bassiana*

| Sl. NO. | Compound                                    | Isolates of Beauveria bassiana | TM          | BR         | B10         |
|---------|---------------------------------------------|---------------------------------|-------------|------------|-------------|
|         |                                             |                                 | +           | -          | +           |
| 1       | 5-Oxotetrahydrofuran-2-carboxylic acid      |                                 |             |            |             |
| 2       | a-D-Glucopyranose, 1,6-anhydro-             |                                 | +           | +          | +           |
| 3       | Undecane                                    |                                 | +           | -          | +           |
| 4       | 2-Deoxy-2-fluoro-1,6-anhydro-a-D-glucopyranose |                               | +           | +          | +           |
| 5       | 3-Hydroxy-2,3-dihydromaltol                 |                                 | +           | +          | +           |
| 6       | 5-Hydroxymethylfurfural                    |                                 | +           | +          | +           |
| 7       | Trioxsalen                                  |                                 | +           | +          | +           |
| 8       | Sucrose                                     |                                 | +           | +          | +           |
| 9       | 3-Deoxy-d-mannoic lactone                   |                                 | -           | +          | +           |
| 10      | 3-Deoxy-d-mannonic acid                     |                                 | -           | +          | +           |
| 11      | n-Hexadecanoic acid                         |                                 | +           | +          | -           |
| 12      | Octadecanoic acid                           |                                 | +           | +          | +           |
| 13      | 9,12-Octadecadienoic acid (Z,Z)-            |                                 | +           | +          | +           |
| 14      | Oleic Acid                                  |                                 | +           | +          | -           |

+ Detected
- Not detected
The enhanced virulence of TM may be attributed to the distinctive metabolites involved in lipid and fatty acid metabolisms. These metabolites might have enabled the fungus to overcome the action of detoxifying enzymes inside insects such as esterases and glutathione-S-transferases which take part in defense responses against the fungus.

In this study, non-targeted profiling approach was performed using GC-MS for metabolite profiling of three isolates of \textit{B. bassiana}. The metabolite profile varied within the species and distinct profiles were recorded in the three study isolates, TM, BR and BbI8. So far, there are no reports on the correlation of metabolites between different isolates of \textit{B. bassiana} and hence the results of the study can be used to interpret the pathogenicity of different isolates of entomopathogenic fungus against any host insect paving way for its management.

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