Efficacy of *Beauveria bassiana* (Balsamo) vuillemin isolates against mango leaf webber, *Orthaga exvinacea* Hampson (Pyridae: Lepidoptera)

Vinayaga Moorthi, P1*, C. Balasubramanian1 and K. Murugan2

1Post Graduate and Research Department of Zoology, Thiagarajar College, Madurai-09
2Professor, Dept. of Zoology, Bharathiar University, Coimbatore.
*For correspondence e-mail: vinayputhu@gmail.com

ABSTRACT

An eco-friendly management of *Orthaga exvinacea*, mango leaf Webber by using *B. bassiana* was carried out in the present investigation. Hydrolytic enzymes, namely chitinolytic and proteolytic enzyme secretions in response to *O. exvinacea* cuticle supplemented medium have been assessed. It was observed that, B1 isolate (Thandikudi) at 10⁸ spore ml⁻¹ was found to be efficient in not only *O. exvinacea* management, but also in enzyme production. The SDS-PAGE analysis confirmed the role of hydrolytic enzymes in pathogenesis of *B. bassiana*. This study could help to identify the possible factors involved during pathogenesis, which could be used as tools to control the lepidopteran pest.

**Keywords**: Chitinase; Proteinase; Beauveria bassiana, Mangifera indica, Orthaga exvinacea.

1. INTRODUCTION

In India, Mango (*Mangifera indica*) is one of the major fruit crop, covers nearly 1.28 million ha area and produced 10.81 million MT fruits with a productivity of 8.44 MT/ha and it is greatly attacked by about 492 species of insects, 17 species of mites and 26 species of nematodes world wide of these 188 species have been reported from India [1, 2]. Among the insect pests, mango leaf Webber, *Orthaga exvinacea* Hampson, (Lepidoptera: Pyralidae) is a major pest responsible for low productivity. The heavily infested trees present a burnt look and severe infestation results in complete failure of flowering [3]. It is widely distributed in different agro-climatic zones of India and has gained the status of serious pest in Uttar Pradesh, Uttarakchhal and Andhra Pradesh states of India [4, 5]. Ninety percent of the completely defoliated/skeletonized shoots, dried and did not fruit in the coming season [6]. There are only few management tools exist which includes pruning of shoots and burning them completely as well as sprays of lambda- cyhalothrin 5 EC (2 ml / lit of water) for the management of mango leaf Webber. With this backdrop, it is an emergency alert to find out the alternate eco-friendly insect pest management tool to enhance the productivity of this fruit crop. In the line of pest control agents, the entomopathogenic mitosporic ascomycete *Beauveria bassiana* (Balsamo) Vuillemin, is an important promising
natural pathogen of insects that have been developed as a microbial insecticide for the management of many major arthropod pests in agricultural, urban, forest, livestock and aquatic environments [7, 8]. The wide utilization of B. bassiana as a bio-insecticide is increased interest in its basic biology, including its fungal secondary metabolites. B. bassiana contains various pathogenesis-related compounds, such as blastospores, hyphal bodies, and metabolites, which are responsible insect-killing process in multiple ways.

The mycelia of this fungus are capable of secreting hydrolytic enzymes such as curtains, protease and lipase, by which it penetrates through these barriers very easily. The secretion will differ according to the characteristic of the integument. Protease is one of the hydrolytic enzymes of entomopathogenic fungi (EPF) which is responsible secretions and is considered to be the virulence factor of EPF. Besides that, chitinase catalyze the hydrolysis of chitin, which is ab 1, 4 linked polymer of N-acetyl-D-Glucosamine and one of the important structural components of insect cuticle. Therefore, such a versatile insect pathogenic fungus was used in the present investigation for the management mango leaf webber, O. exvinacea.

2. MATERIALS AND METHODS

The early instars (I) larvae of O. exvinacea were collected from mango cultivated areas in and around Madurai and were brought to the laboratory, reared in a wooden cage (1x1x1m) with a small mango twig which was inserted in a bottle containing water and tightly closed the water bottle with cotton pad. The larvae were allowed to feed. The II instar larvae were taken for the bioassay studies.

2.1. Isolation of B. bassiana

B. bassiana was isolated from naturally infected insects (Hypothenemus hampei) (Thandigudi, Dindigul district, Tamil Nadu, INDIA) and also from rhizosphere soil samples of different locations, namely Alagar hill of Madurai and Alwarkurichi of Tirunelveli District, Tamil Nadu, India. One gram of soil was diluted with 100 ml of distilled water and was serially diluted. From each dilution, 100 µl was plated on PDA (Hi-Media, India) medium and it was fortified with streptomycin (10 mg/100 mL). It was allowed to grow for 7 days at 27±2°C [9]. After seven days of incubation, the fungal colony was identified. The identified fungal colony was sub-cultured in Sabourd Dextrose Agar (SDA) (Hi-Media, India). The sterilized medium was transferred into sterile Petri dishes (Borosil®) and test tubes (Borosil®) that were then inoculated with conidia by the streak plate method.

All the fungal isolates of the present study were cultured in potato dextrose agar (PDA) (Hi-Media, India). The plates were incubated at 26°C for 10 days. After sporulation, aerial conidia were harvested by flooding the plate with sterile deionized water (d>H2O) containing 0.02% Tween-80. Conidial spore suspensions were prepared and conidial count determined using improved Neubauer haemocytometer. All the suspensions were adjusted to a concentration of 1.5x10⁸ conidia ml⁻¹ from which lower concentrations were prepared by serial dilution technique for bioassay studies.

2.2. Bioassay

The isolates of B. bassiana were used to determine the pathogenicity against O. exvinacea. Pure culture of the test fungi, B. bassiana isolates was grown on sabouraud dextrose agar (SDA) (Hi-Media, India) at 27 ± 2°C until a dense sporulating mat was produced (14 days). The conidial suspension of 10⁸ conidia per ml was prepared by counting the spores in the improved Neubauer counting chamber (Superior Marienfeld, Germany).

The conidial suspension per ml (10⁵ to 10⁸ conidia per ml) was prepared experimental studies. Bioassays with different B. bassiana fungal isolates were carried out by dipping 15 II instar larvae of O. exvinacea in conidial suspensions plus 0.02% Tween 20 at each concentration for 30 s. After 30 s, the larvae was transferred to sterile filter paper and then placed in individual sterilized containers having single mango leaf previously surface sterilized and was cleaned with sterilized paper towels to eliminate excess water. The bioassay setup was conducted in room at 27 ± 2°C at 70 ± 5% RH. Each bioassay per concentration was performed in triplicates. A group of larvae (10 in each replicate) was maintained as control treatment only of distilled water plus 0.02% Tween 20 (Hi-Media, India).

The larvae of O. exvinacea were observed 4, 7, 12 and 16 days after inoculation with each conidial suspension. The dead larvae were placed in a controlled growth chamber to stimulate the development of fungal mycelia and confirm that the death was by infection of the B. bassiana isolates.

Efficacy of B. bassiana (Balsamo) vuillemin isolates against mango leaf webber, Orthaga exvinacea
2.3. Enzyme Assays

*B. bassiana* isolates was grown in a complete medium (0.0001% FeSO₄, 0.05% KCl, 0.15% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.6% NaNO₃, 0.0001% ZnSO₄, 0.15% casein hydrolyzed, 0.05% yeast extract, 1% glucose, 0.2% peptone and 2% agar) being incubated at 28°C for 10 days. In order to carry out, SDS- PAGE protein profile and enzymatic analysis, 10⁷ spores were inoculated in TM medium containing 0.1% bactopeptone, 0.03% Urea, 0.2% of KH₂PO₄, 1.4% (NH₄)₂SO₄, 0.03% MgSO₄·7H₂O, 0.3% C₂H₂O₆ and FeCl₃ as trace element. After three days of incubation, the mycelia was transferred to minimal medium (MM) (0.2% KH₂PO₄, 0.03% MgSO₄, 1.4% (NH₄)₂SO₄ and FeCl₃ as trace element and to MM containing 0.5% *O. exvinacea* dehydrated cuticle (MMC). Both were re-incubated for three days at 29°C in a rotation of 130 rpm on an Orbit incubator shaker (Neolab, Neolab Instruments, Mumbai, India). Both media were double filtered and stored at -20°C for further analysis.

2.3.1. Proteolytic activity

Proteolytic activity from fungi secretion was evaluated according to Leger et al. [10]. Hundred μg of secreted proteins were added in tubes containing 500 μl of 5% casein as a substrate at pH 8.0. Hundred μl of 0.05 M Tris HCl buffer, pH 8.5 was added to the reaction. After incubation for 30 minutes at 40°C, 1500 μl of 5% trichloroaetic acid (TCA) was added to stop enzymatic reaction. A negative control was inactivated enzyme adding TCA before incubating. Samples were centrifuged at 10,000g for 15 minutes at 4°C (Remi, Laboratory Centrifuge, Mumbai, India). The supernatant was measured OD at 280 nm in JASCO V-530 spectrophotometer. Each assay was carried out in triplicate. Casein was served as standard.

2.3.2. Chitinolytic activity

Chitinolytic activity was assayed by measuring the release of reducing saccharides for colloidal chitin as follows [11]. A reaction mixture containing 1 ml of culture supernatant, 0.3 ml of 1M Sodium acetate buffer (SA-buffer) pH 4.7 and 0.2 ml of colloidal chitin was incubated at 40°C for 6-24 hours and then centrifuged at 12,000g for 5 minutes at 6°C (Remi, Laboratory Centrifuge, Mumbai, India). After centrifugation, an aliquot of 0.75 ml of the supernatant, 0.25 ml of 1% solution of Dinitrosalicylic acid (DNS) in 0.7M NaOH, and 0.1 ml of 10M NaOH were mixed in 1.5 ml Eppendorf tubes (Hi-Media, India) and heated at 100°C for 5 minutes. Absorbance of the reaction mixture at 582 nm (A582) was measured after cooling to room temperature in JASCO V-530 spectrophotometer. A calibration curve with N-acetyl-D-Glucosamine (NAGA) as a standard was used to determine reducing saccharide concentration.

2.4. Sodium Dodecyl Sulfate – Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

The chemicals for SDS – PAGE analysis were purchased from Hi-Media, India. The secretions of fungal entomopathogenic fungi such as B1, B2 and B3 was fractionated at SDS-PAGE by using method followed by the discontinuous buffer system of Laemmli [12], which is a modified from Davis [13].

2.5. Statistical Analysis

The total flavonoid content was determined by calorimetric aluminium chloride method Ebrahimzaded et al. (2008) with some modifications. The absorbance was measured at 415 nm using UV-Visible spectrophotometer. A calibration curve was obtained by preparing various concentration of Quercetin in methanol [6].

3. RESULT AND DISCUSSION

3.1. Isolation of *B. bassiana*

The insecticidal efficiency of *B. bassiana* isolates against *O. exvinacea* at various spore concentrations at different days of interval (4th, 7th, 12th and 16th days) were studied and its LC₅₀, LC₉₀ and per cent cumulative mortality obtained on the 16th day after treatment were presented in the table 1. It was observed from the present study that, Bb1 isolate contributed 87.13% mortality while Bb2 and Bb3 revealed 51.36% and 61.75% mortality against *O. exvinacea*. The isolate Bb01 conferred lowest LC₅₀ value (7.6x10⁵) which was followed by Bb03 and Bb02 which represented LC₅₀ values 4.0x10⁶ and 8.2x10⁶ accordingly. A similar result were also observed in our earlier study with different isolates of *B. bassiana* against *Spodoptera litura* and recorded 80.0% mortality by Bb10 isolates at 1.5x10⁸ spore ml⁻¹ followed by Bb09 and Bb02 which resulted in 73.33, 60.0% mortality, respectively [14].

In our another study, *Metarhizium anisopliae* (M2) isolate revealed 100 and 87% mortality against *S. litura* and *Euproctis fraterna* [15]. Similarly, in the present investigation also, 87.13% mortality was recorded against *O. exvinacea*. In our recent study, isolates of *B. bassiana* (Bb08 and Bb10) contributed...
lowest LC₅₀ values (5.9 x 10⁵ and 6.6 x 10⁵) against *Dysdercus cingulatus* [16], which was almost similar to that of the LC₅₀ (7.6x10⁵) recorded in the present investigation. Ambethgar [17] reported that, *B. bassiana* conidial concentration 1x10⁷ spore ml⁻¹ was found pathogenic to *Plocaeders ferrugineus*. It was observed from the present investigation that, the spore concentrations of Bb02 isolate revealed it as promising entomopathogenic fungi in integrated pest management.

**Table 1.** LC₉₀ (Spore mL⁻¹) and LC₅₀ (Spore mL⁻¹) of different isolates of *B. bassiana* during different days of treatment against *O. exvinacea*.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Day</th>
<th>LC₉₀</th>
<th>LC₅₀</th>
<th>% CM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bb01</td>
<td>4</td>
<td>1.9x10⁷</td>
<td>5.1x10⁷</td>
<td>38.53</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1.0x10⁷</td>
<td>3.2x10⁷</td>
<td>48.16</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>5.1x10⁶</td>
<td>3.2x10⁷</td>
<td>58.16</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>7.6x10⁶</td>
<td>1x10⁷</td>
<td>87.13</td>
</tr>
<tr>
<td>Bb02</td>
<td>4</td>
<td>2.0x10⁷</td>
<td>4.9x10⁷</td>
<td>28.04</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2.7x10⁷</td>
<td>7.6x10⁷</td>
<td>38.53</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.0x10⁷</td>
<td>3.6x10⁷</td>
<td>51.36</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>8.2x10⁶</td>
<td>4.4x10⁷</td>
<td>51.36</td>
</tr>
<tr>
<td>Bb03</td>
<td>4</td>
<td>2.7x10⁷</td>
<td>7.7x10⁷</td>
<td>31.06</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>3.3x10⁷</td>
<td>1.1x10⁸</td>
<td>48.16</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.0x10⁷</td>
<td>4.2x10⁷</td>
<td>48.16</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>4.0x10⁷</td>
<td>2.8x10⁷</td>
<td>61.75</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>12.05</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean of three replications; %CM- Values are arc sine transformed, a – d, represents level of treatment, a: Best treatment & d: Poor treatment.

**3.2. Enzyme assay**

The extracellular protease of *B. bassiana* is an essential enzyme in the degradation of cuticle layers of insects [18]. To gain the knowledge of entomopathogenic fungi secreteome in response to mango leaf Webber, *O. exvinacea*, isolates were evaluated and found that B₁ isolate was more virulent than B₂ and B₃ isolates.

In cuticle degradation, protease appears first, followed by chitinase, when *B. bassiana* was grown in culture containing insect cuticle as the sole carbon and nitrogen source [18]. It was observed from the sequential treatment of insect cuticle with protease and chitinase from commercial preparations [19] or from *M. anisopliae* [18], that Chitin hydrolysis by chitinase was enhanced when the cuticle was pretreated with a protease. A similar result was also observed by Murad et al. [20] who treated *Callosobruchus maculatus* with *B. bassiana*. As induction and repression seem to be dependent on the concentration of the amino acid present in the culture medium. Donatti et al. [21] described that the methionine, the amino acid seems to play a regulatory role in Pr1, a protease secretion by *B. bassiana*. In the present study also secretion of hydrolytic enzymes was observed in response to *O. exvinacea* cuticle (Figure 1a and b).

SDS-PAGE analysis of secretions of *B. bassiana* isolate was shown in in figure 2. The result reveals that, there were eight, two and three prominent fractions with respects to B₁, B₂ and B₃ isolates were observed. Furthermore, Leger et al. [22] also determined a major chitinase form of 45 KDa. These results were in accordance with Murad et al. [20] on *B. bassiana* protein pattern 28, 33, 55 and 120 KDa, which has reflected the similar pattern of result observed in the present study (98 KDa, 78 KDa, 66 KDa, 43 KDa, 27 KDa, 20 KDa and 14 KDa bands) (Plate 1). In order to digest the insect cuticle complex completely, protease needed to be in huge amount [23].

In the present study, a little information on the digestion of *O. exvinacea* by the enzymes of these *B. bassiana* isolates was observed. During the infection process of *M. anisopliae*, the subtilisin-like Pr1 protease was the major protein produced and it degrade cuticle much greater than the trypsin-like Pr2 [24]. In the present investigation also, enzyme with greater cuticle degrading efficiency was recorded from Bb1 isolate. Subtilisin-related proteases may therefore have a greater potential for pathogenicity to insects and possibly also for scavenging extracellular proteins, their most likely biological role in saprophyses [25]. Kucera and Samsinakova [26] reported high- and low molecular- weight proteases produced by a strain of *B. bassiana* which supported the present study strongly.

The finding of the present study puts forth a promising entomopathogenic fungus, Bb1 isolates having strong cuticle degrading enzymes suggest and recommend it as one of the important components of the integrated pest management.
**Figure 1a.** Proteolytic activity of *B. bassiana* on Minimal media supplemented with exoskeleton of *O. exvinacea*

![Proteolytic activity graph](image)

**Figure 1b.** Chitinolytic activity of *B. bassiana* on Minimal media supplemented with exoskeleton of *O. exvinacea*.

![Chitinolytic activity graph](image)

**Figure 2.** SDS–PAGE profile of *B. bassiana* secretome on Minimal media supplemented with exoskeleton of *Orthaga exvinacea*

![SDS–PAGE profile](image)

*Efficacy of *B. bassiana* (Balsamo) vuillemin isolates against mango leaf webber, *Orthaga exvinacea*
4. CONCLUSION

The pathogenicity and its associated enzyme production in the presence of cuticular substrates could provide information about possible factors involved during pathogenesis. The results presented in this study increase the knowledge about protease production in *B. bassiana* opening new avenues for the virulence against the mango leaf webber *O. exvinacea* during the infection process. Molecular studies would be addressed to predict gene expression corresponding to enzymes, which helps to understand the role of different carbon sources on their regulation.

Acknowledgement

The authors thank the President, Principal, and Head of the department of Zoology, Thiagarajar College, Madurai for providing necessary facilities to work. The authors also thank Dr. D. Kodi Munthiri and Dr. P. Tharmaraj for providing permission to use the UV spectrophotometer. Author PVM thanks, UGC-RGNF, New Delhi for their financial support.

References


