Scanning electron microscopic studies of Beauveria bassiana against Lipaphis erysimi Kalt

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Received: July 4, 2016; Revised received: December 15, 2016; Accepted: February 14, 2017

Abstract: This work was aimed to identify the LC<sub>50</sub> of the indigenous fungal isolates for controlling L. erysimi infestation in mustard aphid besides to probe the mechanism of action of the local isolates and comparison of the efficacy with the reference culture and commercial formulation 'Mycojaal'. Three isolates of entomopathogenic fungi Beauveria bassiana were tested for infection on nymph of Lipaphis erysimi Kalt. using scanning electron microscopy (SEM) to record any variation. The SEM revealed adhesion of spores of B. bassiana followed by penetration of L. erysimi nymph surface. It was observed that all Beauveria isolates showed little variation with respect to penetration and adhesion at different time intervals. Further, lethal concentration (LC<sub>50</sub>) values of B. bassiana isolates against L. erysimi was recorded and was lowest (0.05x10<sup>7</sup> spores/ml) in native isolate F10 after 120 hours of treatment. The study has established the need for the isolation and evaluation of the indigenous Beauveria isolate. Moreover, it also exhibited the efficacy of the reference and commercially available biocontrol agents.

Keywords: Beauveria bassiana, Lipaphis erysimi, Lethal concentration, Scanning electron microscopy

INTRODUCTION

Mustard aphid (Lipaphis erysimi Kalt.) (Homoptera, Aphididae) is a major pest of brassica crops in subtropics and tropical regions. It feeds by sucking the sap of their host-plants and leads to leaf curling, shrewling and yellowing, which affects the leaf size and yield. Mustard aphid may damage the crop from seedling stage to maturity, with highest population occurring during the flowering and podding stages. It is responsible for causing the yield losses ranging from 35.4 to 96 per cent (Sahoo, 2012). The chemical insecticides used to manage this pest cause environmental and health problems apart resulting in development of resistant. Indiscriminate use of synthetic chemical pesticides in crop protection causes several socio-economic problems throughout the world. Harrington and Emden (2007) have shown development of resistance in mustard aphid to a number of carbamate, pyrethroid and organophosphate based insecticides. Aphid resistance to common insecticides has stimulated interest in developing alternative methods for its control for sustainable crop practices (Gurtet et al., 2004).

Entomopathogenic fungi are important biocontrol agents which play a vital role in pest management (Cooke, 1977). Various entomopathogenic fungi such as Lecanicillium sp. (Jung et al., 2006), B. bassiana (Quesada-Moraga et al., 2006), M. anisopliae, Isaria sp. and Nomuraea sp. (Devi et al., 2003) have been used for the management of insect pests. Among these entomopathogenic fungi, Beauveria species are the most promising biocontrol agents and are known to attack a wide range of insect pests. Worldwide, B. bassiana parasitizes arthropod species, causing white muscardine disease. This fungus rarely infects humans or other animals, which makes it a safer biocontrol agent for management of crop insects (Ormond et al., 2010). Keeping this in mind, the present study was conducted to compare pathogenicity of three Beauveria isolates against nymph of L. erysimi and the SEM studies were performed to probe the mechanism of action of the local isolates as compared to commercial and reference strains.

MATERIALS AND METHODS

Source of Beauveria bassiana: Three Beauveria bassiana isolates, viz., B. bassiana MTCC 4495 (procured from IMTECH, Chandigarh), one native B. bassiana isolate F10 (isolated from soil sample by Galleria bait method) and one isolated from commercial formulation (Mycojaal, Pest control India, Bangalore) were evaluated in the present study. These isolates were maintained on Sabouraud Dextrose Yeast Extract Agar (SDYA) medium at refrigeration temperature.

Preparation of culture suspension: The SDYA medium was inoculated with the B. bassiana isolates and
incubated at 25±2 °C in Erlenmeyer flasks under static conditions. After ten days of incubation the aqueous spore suspensions were prepared. The conidia were harvested by scratching and suspended in sterilized 0.1 % Tween 80 solution (Luz et al., 1998).

**Collection of L. erysimi and bioassay:** Adults and nymphs of *L. erysimi* were collected from mustard field and reared on mustard plants in the laboratory. These nymphs were reared on mustard shoots and leaves under ambient environmental conditions at 25±2 °C. The nymphs of the next generation were used in present study.

Three *B. bassiana* isolates along with untreated control were evaluated against *L. erysimi*. There were four replications and thirty nymphs per replica used for the study. Mustard leaves along with shoots were treated with *B. bassiana* suspension by leaf dip method. Wet filter paper disc (8.5 cm, Whatman grade) was placed on the bottom of each petri plate before placing treated leaves. A rectangular paraffin was placed under the wet cotton was used to provide water to the leaves as to avoid over-moistening of the filter paper. Thirty aphids were released per replica and the plates were incubated in BOD humidifier at 25±2 °C. The petri plates were observed to record the nymphal mortality. The experiment was performed using completely randomized design and the data was analyzed with LC$_{50}$ in POLO statistical software.

**Preparation of sample for Scanning electron microscopy:** The SEM studies of fungal isolates of *B. bassiana* were performed on nymphs of mustard aphids. Conidial suspension of *B. bassiana* at 10$^5$ conidia ml$^{-1}$ was sprayed on nymph stage of *L. erysimi* after 24, 48, 72, and 120 hours of incubation respectively. Three to five samples were taken out and processed for SEM studies by using the method of Talaei-hassanloui et al. (2007) with little modifications.

The intact larvae samples were fixed in 2.5 % glutaraldehyde for 24 hrs at 4°C followed by rinsing of the sample thrice with Reagent A (0.1 M cacodylate buffer) for 15 minutes at 4 °C. The secondary fixation of larvae was done with Reagent B (1 % osmium tetroxide prepared in 0.2 M cacodylate buffer) at 4 °C for 1 hour. The samples were again washed thrice with Reagent A at 4 °C to rinse unixed osmium tetroxide. Then the dehydation of the sample was done by passing it through Reagent C involving an ascending ethanol series. The processed nymphs were stubbed and sputter coated in Hitachi gold sputter coater (model E1010) to view in secondary electron imaging mode in Hitachi S-3400N Scanning Electron Microscope for studying the activity of fungal isolate on infected nymph.

### RESULTS AND DISCUSSION

**Scanning electron microscopy of Beauveria bassiana against Lipaphis erysimi KALT:** The SEM is one of the most convenient tools to observe the mode of action of entomopathogenic fungi, so that colonization capabilities and extent in rate of infection of the host can be determined (Sun et al., 2016). This tool was used in the present study to investigate the extent and rate of growth of *B. bassiana* on nymphs of *L. erysimi* after first, third, fourth and fifth day of inoculation, so as to compare the biocontrol efficacy of *B. bassiana* MTCC 4495, *B. bassiana* F10 (Local isolates) and Mycojaal (commercial product of *B. bassiana*) along with untreated control. After 24 hrs of inoculation no fungal growth was observed in *B. bassiana* MTCC 4495, local fungal isolate F10 and untreated control whereas, commercial formulation isolate Mycojaal showed fungal hyphae growth on the exoskeleton of aphid (Fig. 1). Similar to our results, Li et al. (2016) have observed the adsorption of the conidia and hyphal growth on the surface of the *Solenopsis invicta* Buren after 2 and 20 hours of inoculation with 1×10^8 conidia ml$^{-1}$ of *B. bassiana* strain ZGNKY-5. After 72 hours of inoculation, the process of conidiogenesis was observed in Mycojaal. However, penetration of *L. erysimi* after 24, 48, 72, and 120 hours of incubation respectively. Three to five samples were taken out and processed for SEM studies by using the method of Talaei-hassanloui et al. (2007) with little modifications.

<table>
<thead>
<tr>
<th>Fungal Isolates</th>
<th>Observations (in hrs)</th>
<th>LC$_{50}$ (x10$^5$ conidia ml$^{-1}$)</th>
<th>FL</th>
<th>Slope</th>
<th>Chi-square</th>
<th>Heterogeneity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTCC4495</td>
<td>96</td>
<td>0.05</td>
<td>0.00-9.087</td>
<td>0.157±0.042</td>
<td>0.789</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.05</td>
<td>0.619-5.86</td>
<td>0.349±0.046</td>
<td>5.80</td>
<td>1.93</td>
</tr>
<tr>
<td>F10</td>
<td>96</td>
<td>0.10</td>
<td>0.007-0.152</td>
<td>0.388±0.054</td>
<td>2.89</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.11</td>
<td>0.004-0.56</td>
<td>0.444±0.057</td>
<td>5.89</td>
<td>1.96</td>
</tr>
<tr>
<td>Mycojaal</td>
<td>96</td>
<td>0.04</td>
<td>0.00-0.004</td>
<td>0.253±0.50</td>
<td>0.20</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.06</td>
<td>0.00-0.146</td>
<td>0.44±0.172</td>
<td>0.43</td>
<td>0.15</td>
</tr>
</tbody>
</table>

**Table 1.** The lethal concentration (LC$_{50}$) of fungal isolates against *L. erysimi*. 

Fig. 1. Scanning EM study of aphid post 24 hours of inoculation A) Fungal hyphae growth on the exoskeleton of aphid in Mycojaal treatment; B) Absence of fungal growth in fungal isolate F 10; C) Untreated control.
simicuticle by elongated hyphae in *B. bassiana* MTCC 4495 was recorded. A similar result was observed in the form of network of hyphae growing out of the nymph head creating a hollow crescent in the head region for F10 (local isolate of *B. bassiana*) post 72 hours of inoculation and incubation (Fig. 2). The hyphae must have ramified inside the nymph body by generation of secondary hyphae due to germination of spores, formation of appressorium followed by penetration of germ tube in the cuticle. Li et al. (2016) reported formation of the germ tubes of the conidia oriented toward the cuticle after 48 h. The primary vegetative hyphae exhibited formation of elongated chains of two or more conidia as observed under SEM but in untreated control no fungal growth was observed.

After 96 hours of inoculation, mycelium network of Mycojaal isolate covered the whole body of nymph whereas, the presence of conidia, appressorium and germ tube on cuticular surface of *L. erysimi* was observed in *B. bassiana* MTCC 4495 treated nymphs (Fig. 3). After 120 hours of inoculation and incubation, thick mycelium network covering the outer surface of nymph was also observed in treatment involving inoculation with MTCC 4495 in *L. erysimi* (Fig. 4). Hyphal ramification was recorded throughout the internal surface followed by invasion of outer cutin exoskeleton by the secondary hyphae coming out of the insect internal tissue in fungal isolate F10 after 120 hours. So, little but variation in pathogenesis of *Beauveria* isolates was recorded at different time interval of fungal infection. A similar SEM study showed variation in efficiency and extent of infection among fungal isolates of *B. bassiana* than *M. anisopliae* while little variation was recorded in timing and duration of the phases in the infection process for these two isolates (Moino Jr. et al., 2002). Similar to our observations, Gabarty et al. (2014) reported adhesion and penetration by *B. bassiana* and *M. anisopliae* on *Agratisipsilla* larvae by SEM. The SE micrographs showed presence of dense hyphal network as well as spores on insect cuticle and also in the body cavity of infected larvae.

Moguel et al. (2013) showed the infection process by *Isaria fumosorosea* EH-506/3 colonizing whitefly (*Trialeurodes vaporariorum*) nymphs using light and scanning electron microscopy. The fungal growth development index was used to determine pathogenicity of the test isolates. They recorded colonization of *T. vaporariorum* by the fungus due to formation of cuticular penetration structures and reported that *I. fumosorosea* produced structures that resembled appressoria in shape causing serious cuticular damage probably via an enzymatic action and recorded that isolate EH-506/3 was suitable for whitefly biocontrol.

Another SEM report by Talaei-hassanloui et al. (2007) compared the relative efficacy of the uni-, bi- and multi-directional conidial growth i.e. differential polar growth in *B. bassiana* for imparting pathogenicity. They observed that the unidirectional germination of conidia produced stronger germ tubes and appressorium like structures and thus had highest infection potential as compared to bidirectional and multidirectional conidial growth. Our results also exhibit similar unidirectional germination of the conidia in the local isolate F-10 and the reference culture MTCC 4495.

**Lethal concentration (LC50) against nymphs of *L. erysimi***: The conidial count required to kill 50 per cent (LC50) population of nymph stage of *L.erysimi* was
The indiscriminate use and over-application of pesticides have serious environmental toxicity concerns which may lead to development of pest resistance, bio-magnification, and harmful impact on non-target organisms. The present study was undertaken to compare and assess the efficacy of three different isolates of entomopathogenic fungus B. bassiana. The results clearly exhibited little but variation in the extent of infection depicted in terms of spore germination in the aphid cuticle post inoculation. The LC<sub>50</sub> also varied being lowest (0.04) for commercial formulation 96 hr post inoculation while highest (0.11) for native isolate F10 post 120 hours of inoculation.

**ACKNOWLEDGEMENTS**

The authors are thankful to the Head, Department of Entomology and Incharge, Electron Microscopy and Nanoscience Laboratory, Department of Soil Science, Punjab Agricultural University, Ludhiana, Punjab, India for providing the necessary facilities to carry out the present research.

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**Fig. 4. Scanning EM study of aphid surface post 120 hours of inoculation A) Fungal hyphae growth covering the outer surface in Mycojaal treatment; B) Formation of thick hyphal network on the surface of L. erysimi KALT on inoculation with B. bassiana MTCC 4495; C) Hyphal ramification throughout the internal surface followed by invasion of outer chitin exoskeleton by the secondary hyphae coming out of the insect internal tissue in fungal isolate F10; B) Untreated control.**

Calculated for all the three B. bassiana isolates. The dosage mortality data obtained was subjected to probit analysis and LC<sub>50</sub> values of each fungus was calculated post 96 and 120 hours of inoculation. The LC<sub>50</sub> values of fungal isolates of L. erysimi ranged from 0.04 to 0.10 after 96 hours of treatment. The highest LC<sub>50</sub> value (0.10 x 10<sup>7</sup> spores ml<sup>-1</sup>) was recorded in native fungal isolate F10 which showed low effectiveness against L. Erysini whereas, lowest LC<sub>50</sub> value (0.04 x 10<sup>7</sup> spores ml<sup>-1</sup>) was found in commercial isolate of B. bassiana (Mycojaal) (Table 1).

After 120 hours of treatment, highest LC<sub>50</sub> value (0.11 x 10<sup>7</sup> spores ml<sup>-1</sup>) was recorded in native fungal isolate F10 and lowest LC<sub>50</sub> value (0.05 x 10<sup>7</sup> spores ml<sup>-1</sup>) was recorded in MTCC 4495. Mycojaal isolate and MTCC 4495 were observed to be most effective against L. erysimi KALT. Similarly, other scientists also worked on LC<sub>50</sub> value of different fungal isolates against different insect pests and reported that the fungal isolates with lowest value of LC<sub>50</sub> were more pathogenic. Jiji et al. (2006) evaluated B. bassiana for its potential to control Bactrocera dorsalis and recorded LC<sub>50</sub> values of 7.0 x 10<sup>3</sup>, 2.0 x 10<sup>5</sup>, 5.0 x 10<sup>6</sup> spores ml<sup>-1</sup> on 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> day of inoculation, respectively. Similarly, Alizadeh et al. (2007) studied the effect of some B. bassiana isolates on Agonosma pistaciae with LD<sub>50</sub> value of 3.91 x 10<sup>2</sup> and 3.63 x 10<sup>4</sup> spore per insect.

**Conclusion**

The indiscriminate use and over-application of pesticides have serious environmental toxicity concerns which may lead to development of pest resistance, bio-magnification, and harmful impact on non-target organisms.


