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Scanning electron microscopic studies of *Beauveria bassiana* against *Lipaphis erysimi* Kalt

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Abstract: This work was aimed to identify the LC_{50} 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_{50}) values of *B.bassiana* isolates against *L. erysimi* was recorded and was lowest ($0.05x10^7$ spores/ml) in *B. bassiana* MTCC 4495 and highest ($0.11.X10^7$ spores/ml) was recorded 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, shrivelling 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 (Gurr*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 pathogencity 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 ^{0}C in Erlenmeyer flasks under static conditions. After ten days of incubation the aqueous spore suspensions were prepared. The conidia were harvested by scrapping 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 ^{0}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, Whattman grade) was placed on the bottom of each petri plate before placing treated leaves. A rectangular paraflim 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 ^{0}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 nymph of mustard aphids. Conidial suspension of *B. bassiana* at 10^9 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 tetra oxide 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 unfixed osmium tetra oxide. Then the dehydration 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.

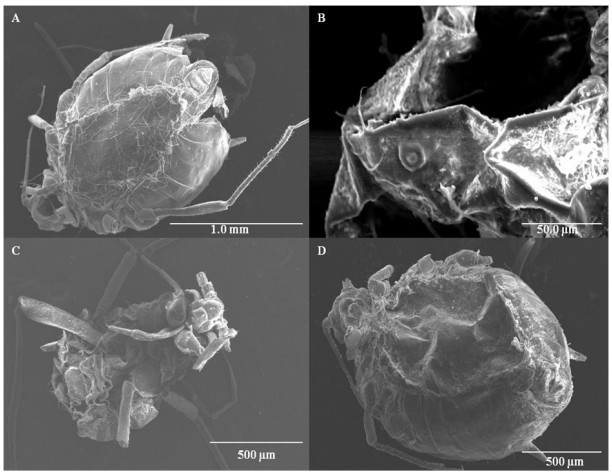

Fig. 1. *Scanning EM study of aphid post 24 hours of inoculation A) Fungal hyphae growth on the exoskeleton of aphid in Mycojaal treatment; B)Absence of fungal growth in MTCC 4495; C) No fungal growth observed in fungal isolate F 10; C) Untreated control.*

RESULTS AND DISCUSSION

Scanning electron microscopy of *Beauveria bassiana* against *Lipaphis erysmi* 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. ery-*

Table 1. The lethal concentration (LC_{-50}) of fungal isolates against *L. erysimi.*

Fungal Isolates	Observations (in hrs)	LC_{50} (x10^{7}spores ml^{-1})	FL	Slope	Chi -square	Hetrogenity
MTCC4495	96	0.05	0.00-9.087	0.157±0.042	0.789	0.78
	120	0.05	0.619-5.86	0.349±0.046	5.80	1.93
F10	96	0.10	0.007-0.152	0.388±0.054	2.89	0.97
	120	0.11	0.004-0.56	0.444±0.057	5.89	1.96
Mycojaal	96	0.04	0.00-0.004	0.253±0.50	0.20	0.07
	120	0.06	0.00-0.146	0.44±0.172	0.43	0.15

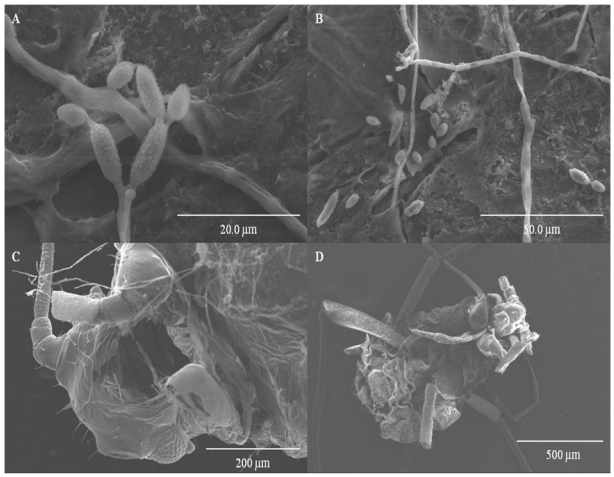

Fig. 2. *Scanning EM study of aphid post 72 hours of inoculation A) Presence of conidia (conidiogenesis) on surface of aphid Mycojaal treatment; B) Cuticle penetration by elongated hyphae in MTCC 4495; C) Dislocation of the chitin plates of head showing creation of hollow cavity inside head due to ramification of hyphae and secretion of tissue dissolving enzyme by B. bassiana F10 isolate inside nymph; D) Untreated control.*

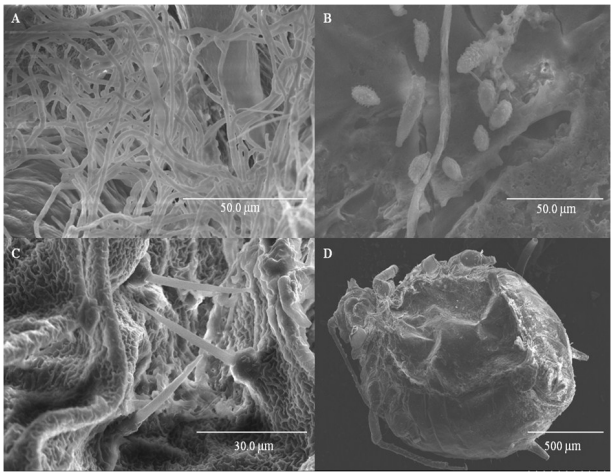

Fig. 3. *SEM study of aphid surface post 96 hours of inoculation A) Mycelium network covering the body of nymph in Mycojaal B) Presence of conidia appressorium and germ tube on cuticular surface of L. eysimi in MTCC 4495 C) Growth of B. Bassianaisolate F10 on the surface of nymph ;D) ntreated control.*

simi cuticle 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 chitin 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. anisoplae* 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 *Agrotis ipsilon* 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 enzymaticaction 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 (LC_{50}) against nymphs of *L. erysimi*: The conidial count required to kill 50 per cent (LC_{50}) population of nymph stage of *L.erysimi* was

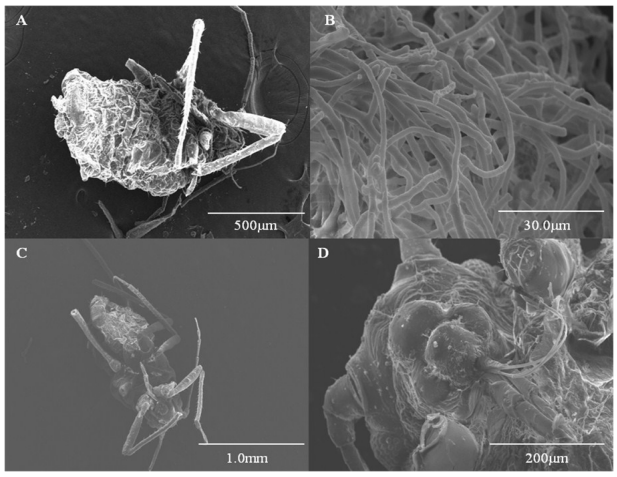

Fig. 4. *Scanning EM study of aphid surface post 120 hours of inoculation A) Fungal hyphae growth covering the outer surface in Mycojaal treatment; B) Formation of thick hyphal network on the surface of L. eysimi KALT on inoculation with B. bassiana MTCC 4495; C) Hyphal ramification throughout the internal 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_{50} values of each fungus was calculated post 96 and 120 hours of inoculation. The LC_{50} values of fungal isolates of *L. erysimi* ranged from 0.04 to 0.10 after 96 hours of treatment. The highest LC_{50} value (0.10×10^7 spore ml^{-1}) was recorded in native fungal isolate F10 which showed low effectiveness against *L. Erysimi* whereas, lowest LC_{50} value (0.04×10^7 spores ml^{-1}) was found in commercial isolate of *B. bassiana* (Mycojaal) (Table 1).

After 120 hours of treatment, highest LC_{50} value (0.11×10^7 spores ml^{-1}) was recorded in native fungal isolate F10 and lowest LC_{50} value (0.05×10^7 spores ml^{-1}) 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_{50} value of different fungal isolates against different insect pests and reported that the fungal isolates with lowest value of LC_{50} were more pathogenic. Jiji *et al.* (2006) evaluated *B. bassiana* for its potential to control *Bactrocera dorsalis* and recorded LC_{50} values of 7.0×10^8, 2.0×10^7, 5.0×10^6 spores ml^{-1} on 3rd, 4th and 5th day of inoculation, respectively. Similarly, Alizadeh *et al.* (2007) studied the effect of some *B. bassiana* isolates on *Agonoscena pistaciae* with LD_{50} value of 3.91×10^2 and 3.63×10^4 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, biomagnification, 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_{50} 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.

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