Microscopic investigation to determine the effect of *Beauveria bassiana* (Bals.) Vuill. and *Cymbopogon citratus* (DC.) Stapf. treatment on different life stages of *Musca domestica* (L.)

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Abstract Microscopic investigation was done to determine the effect of entomopathogenic fungi, *Beauveria bassiana* and essential oil of *Cymbopogon citratus* on different life stages of *Musca domestica*. Scanning electron microscopy investigation of fungal infected larvae showed sluggish movement, rigor, and failure of body to balance in water. Treated larvae also revealed varied level of cuticle shrinkage and extreme dehydration. Surface of *B. bassiana* infected pupae showed varied stage of mycelial growth, while the cadaver of adult fly was observed to have extensive fungal growth covering their entire body surface. The application of *C. citratus* oils on *M. domestica* larvae resulted in skin shrinkage, spinous cells proliferation and bleb formation, while the treated pupae showed high incidence of incomplete emergence and malformation in emerged adult flies. The current study establishes effect of *C. citratus* essential oil and *B. bassiana* infection on different life stages of *M. domestica*.

Keywords *Musca domestica* · *Beauveria bassiana* · *Cymbopogon citratus* · Microscopy · Life stages

Introduction

In recent years, the interest in biological methods for insect control has grown considerably due to rising insecticide resistance among insects from the excessive use of

chemical insecticides (Mishra and Malik 2012). Biological control agents, such as entomopathogenic fungi and essential oils, have shown a considerable potential for control of various insects population, with several of them being considered for commercial application. Entomopathogenic fungi with their low mammalian toxicity and natural prevalence among insect/pests, while essential oils with its reported efficacy against various pests, target specificity and safety to human beings, are being considered as good control option. *Musca domestica* L. (Diptera: Muscidae) or common house fly, is an important vector of medical, veterinary and domestic significance (Mishra et al. 2011). They are one of the most widely dispersed non biting insects in the world, which belongs to a group of domestic flies often called “filth flies”. Their association with more than 100 pathogens of medical and veterinary significance, as well as proximity to human dwellings and animal farms has become a serious concern, requiring immediate attention for their control (Malik et al. 2007).

Beauveria bassiana (Bals.) Vuill., an entomopathogen with extremely broad host range has been successfully used as commercial biopesticide for the control of various agricultural insects (Pirali-Kheirabadi et al. 2007). Although, there have been relatively fewer reports implicating application of *B. bassiana* against insect vectors, studies documenting its efficacy against *M. domestica* have been established (Watson et al. 1995; Lecuona et al. 2005; Mishra et al. 2011). Similarly, insecticidal activity of *Cymbopogon citratus* (DC.) Stapf., commonly known as lemon grass, is well documented for the control of various agricultural and non-agricultural insects (Ishii et al. 2010; Hanifah et al. 2011). Among vectors, activity of *C. citratus* oil has been demonstrated against mosquito larvae (Cavalcanti et al. 2004; Amer and Mehlhorn 2006). The insecticidal activity of *C. citratus* essential oil against *M.*

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domestica was reported by Kumar et al. (2013), which established the efficacy of oil and its monoterpenes as an excellent larvicidal and pupicidal agent against *M. domestica*.

The mechanism through which *B. bassiana* or any other entomopathogenic fungi acts on host insect could be divided into three parts: adhesion of the fungal spore, penetration through the cuticle, and establishment within the host (Mishra et al. 2013, 2015). Attachment of conidia to insect's epicuticle is accompanied by electrostatic and hydrophobic interactions. Subsequent to the attachment, germination of fungal conidia occurs on insect cuticle, followed by penetration of the germinating conidia into the cuticle. After penetration, fungus grows in the host haemolymph, taking up nutrients, producing toxins, destroying host cells and eventually killing the insect (Anderson et al. 2011). However, the mode of action of essential oils or their constituents, as insecticides is not clearly known. The essential oils have been reported to affect insect physiology in diverse ways; affecting biochemical processes, endocrinological balance, blocking of acetylcholinesterase synthesis thus disrupting neuromuscular co-ordination, or manipulation of octopamine receptor to increase cAMP production (Rattan 2010; Kumar et al. 2011). In the present study, attempts have been made to study the effect of fungal infection and treatment of essential oil on outer surface of *M. domestica*. The infection mechanism of *B. bassiana* to larvae and pupae of *M. domestica* was studied through scanning electron microscopy (SEM) analysis, while infected adults were evaluated using phase contrast microscopy. The mechanism of action of *C. citratus* to *M. domestica* was elucidated in terms of changes in external morphology of the treated larvae using SEM characterization.

Materials and methods

Musca domestica

Adult house flies were collected from the garbage site of the Indian Institute of Technology, Delhi, India by sweep net method. The field collected flies were reared in cylindrical boxes containing a diet of groundnut oil cake and wheat bran (1:3), according to the method described by Kumar et al. (2011). Hatched larvae were transferred individually to cylindrical vials (28 × 12 mm) containing a larval diet (constituents: 2 g groundnut oil cake, 5 g wheat bran, 2 g milk powder, 1 g honey mixed with 10 mL of water) which was changed daily until larvae reached to the pupal stage.

Essential oil

Essential oil of *C. citratus* was purchased from the Kanta Chemicals Pvt. Ltd, Khari Bowli, New Delhi, India, and was stored in plastic bottles at 4 °C.

Fungal culture

Beauveria bassiana HQ917687 used in the present study was isolated in our laboratory from soil samples collected from Northern part of Uttar Pradesh, India and characterized for its morphological and cultural characteristics (Capek and Fassati 1977). The efficacy of fungal isolate against house fly has been reported earlier by Mishra and Malik (2013). The fungal isolate was maintained on Potato Dextrose Agar slants at 4 °C.

Preparation of conidial suspension

Conidial suspension of *B. bassiana* was prepared by harvesting the conidia from 5 day old slant culture by adding 10 ml of 0.1 % sterile Tween 80, vortexing for 5 min, and filtering through a sterilized 8 µm membrane filter disk. The filtered conidial suspension was enumerated (1×10^8 conidia/ml) and checked for viability using an Automatic Cell Counter (Cellometer[®] Vision HSL, Nexcelom Bioscience), and used for infection of different life stages of *M. domestica* according to the method described by Mishra and Malik (2013).

Microscopic analysis

Fungus infected/*C. citratus* oil treated dead larvae, and live larvae from control were prepared for SEM visualization using the method described earlier by Kumar et al. (2012a). For SEM analysis, larvae/pupae of *M. domestica* were primarily fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer for 1–2 h. Thereafter, it was washed with distilled water for 20 min, followed by secondary fixation with 1 % osmium tetroxide (OsO₄) in distilled water for 2 h. Secondary fixation was followed by washing with distilled water (20 min) and then serial dehydration with 45, 60, 75, 90, and 100 % ethanol for 10 min each. After dehydration, samples were subjected to critical point drying (CPD) in hexamethylene disilazane (HMDS). All the processes were performed at room temperature. Samples were mounted on silver stub for SEM observation and gold-covered by cathodic spraying (Polaron gold). Observation was made on a scanning electronic microscope (ZEISS EVO 50) under the following analytical condition: EHT ¼ 20.00 kV, WD ¼ 9.5 mm, Signal A ¼ SE1.

Fig. 1 SEM micrograph of *M. domestica* larvae after *B. bassiana* treatment **a** Entire larvae, **b** intersegmental region, **c** anterior region; *M. domestica* larvae after treatment with *C. citratus* essential oil, **d** entire larvae, **e** intersegmental region, **f** anterior region

For adult flies of *M. domestica*, the image analysis of fungal infected dead cadaver was done through phase contrast microscopy (Leica microscope DM2500). The magnification of observation was kept at 40 $\times$. The analysis was performed 5 days post exposure of fungal conidia to host insect.

Efficacy of *B. bassiana* against house fly larvae and pupae under simulated field condition

The assay to evaluate efficacy of *B. bassiana* against *M. domestica* larvae and pupae was performed on the decaying waste matrix to simulate the field condition (Mishra et al. 2013). The waste matrix (2 kg) was placed in plastic trays (75 $\times$ 60 cm) and saturated with 40–50 % moisture. After 12 h, the *B. bassiana* spore

suspensions (1×10^6 conidia/g) was added to the matrix and mixed thoroughly. In one set of trays, 200 *M. domestica* larvae (2nd instar) were added, while in another set, 200 pupae were added, after 24 h of fungal inoculation. A parallel control set of trays (without *B. bassiana* inoculation) was prepared in the similar fashion with 200 *M. domestica* larvae and pupae, respectively. Each set of experiments were done in triplicate. The trays were kept at room temperature within a netted cage (80 $\times$ 65 $\times$ 60 cm) for the next 10 days. For the larval assay, the formation of pupae and emergence of flies were observed. The number of pupae formed or flies emerged were counted together and rest of the larvae, which could not be accounted for, were considered as dead. For the assay against *M. domestica* pupae, the effect of *B. bassiana* was assessed in terms of percentage

Fig. 2 SEM micrograph of untreated *M. domestica* larvae **a** Entire larvae, **b** intersegmental region, **c** anterior region [reproduced from Kumar et al. (2012b)]

of pupae failing to develop into adult fly (suppression of adult emergence). Further, the adult flies emerged from treated pupae were analysed for fungal infection.

Results and discussion

SEM image of *M. domestica* larvae treated with *B. bassiana* and *C. citratus* essential oil is represented in Fig. 1. The visual analysis of *M. domestica* larvae treated with *B. bassiana* showed sluggish movement, rigor, and failure of body to balance in water. SEM image analysis of treated larvae revealed shrunken cuticle and extreme dehydration. The treated larvae also showed fungal growth on their body surface, which was present as mycelium or conidial aggregates (Fig. 1a–c). The larvae treated with *C. citratus* essential oil showed skin shrinkage and distortion with protruding intersegmental regions (Fig. 1d). Figure 1e highlights an intersegmental region of the treated larva, revealing shrinkage of intersegmental region along with proliferation of spinous cells and bleb formation. Prominent bleb formation was also observed in the anterior region of the treated larva (Fig. 1f). In contrast, the control larvae had normal and intact cuticle, smooth and well formed body parts and showed normal body movement (Fig. 2). Insun et al. (1999) investigated the effect of *Kaempferia galanga* on mosquito larvae through SEM. They reported convulsion, tremor, unnatural positioning, incoordination, rigor and sluggish movement in the treated larvae, which was analogous to the present study. Shrinkage of treated larvae was attributed to the destruction of irregular ridge like reticulum present on the larval surface (Insun et al. 1999). Sukontason et al. (2004) reported deformation in the integument and intersegmental spines in the eucalyptol treated house fly larvae, while Kumar et al. (2012b) reported proliferation of spinose cells and bleb formation in the *M. domestica* larvae treated with *Mentha × piperita* essential oil. Further, *M. domestica* larvae treated with *Eucalyptus globulus* essential oil showed skin shrinkage especially at spinose rings with protruding intersegmental regions (2012c). The above reports were in confirmation with the results of the present study.

SEM image analysis of *M. domestica* pupae treated with *B. bassiana* is depicted in Fig. 3. The longitudinal view of *M. domestica* pupae showed intersegmental spines between different thoracic segments (Fig. 3a). Surface of the treated pupae showed varied stage of mycelial growth and few fungal spores. Mycelial growth was in dry condition due to the treatment procedure followed for SEM visualization. Application of *C. citratus* essential oil on *M. domestica* pupae resulted in high incidence of incomplete emergence and malformation in emerged adult flies in terms of comparatively reduced size and stunted growth. In cases where suppression in adult fly emergence was observed, treated pupae showed either visible shriveling or distortion of external cuticle surface, or no change in external surface of pupae. This

Fig. 3 SEM micrograph of *B. bassiana* treated house fly pupae **a** longitudinal view of house fly pupae, **b** magnified surface of pupae showing fungal growth, **c** fungal growth on pupal surface

may be due to the inherent mode of action, through which essential oils act against insects.

Figure 4 represents microscopic image of adult flies after treatment with *B. bassiana*. The images reveal growth of fungal mycelia over different body parts of infected flies. The fungus was seen to cover entire outer body surface of dead cadaver. The growth of fungal mycelium on surface of adult fly was extensive compared to the fungal growth observed on the larval/pupal surfaces. The observation of fungus mycelium on dead cadaver confirms with established theory for fungal infection mechanism. Although, *C. citratus* oil have been reported to be highly effective against *M. domestica* adult for repellency and fumigation assays (Kumar et al. 2011, 2013), their contact application against adult flies could not be done due to viscous nature of essential oils. Therefore, the effect of *C. citratus* oil on adult flies through study of microscopic images was not attempted. Similar to the present study, Wakefield (2006) observed fungal conidia and germ tubes on the cuticle surface of storage beetle (*Oryzaephilus surinamensis* and *Tribolium confusum*) treated with *B. bassiana*. SEM observation of the cuticle surface showed higher number of adhered and germinating conidia on *O. surinamensis* than *T. confusum*. This was attributed to the presence of greater number of

setae in *O. surinamensis* which may have assisted the adherence of conidia. Further, these setae are known to favour the conidial germination by providing optimum moisture level. The importance of setae in fungal infection as suggested by Wakefield (2006) may explain high fungal infections to adult flies and low infection to larvae observed in this study. Larvae of *M. domestica* due to absence of any setae like structure would be less likely to support conidial adherence or germination, thus resulting in low *B. bassiana* infection (36.7–72.3 %) as observed in our earlier study (Mishra and Malik 2012). On the other hand, abundance of setae on adult flies supports and propagates any infection from entomopathogenic fungi resulting in relatively high mortality of adult house flies (72.7–100 %). The importance of setae in infection propagation could be explained more clearly from the kinetic of *M. domestica* mortality (Fig. 5). Infection of *B. bassiana* to adult flies was very low in the beginning of the treatment (0–1 days) due to time required for establishment and adherence of conidia. However, once established, conidia germinate and propagate infection to the other flies, resulting in drastic increase in mortality (2–3 days). Afterwards, a constant mortality for adult flies could be observed due to fungal infection. Similar trend is seen with *B. bassiana* infection to the *M. domestica*

Fig. 4 *B. bassiana* infection in adult houseflies showing growth of fungal mycelium on cadaver, **a** leg, **b** joint between leg and abdomen, **c** leg joint between tibia and tarsus, **d** leg joint between femur and tibia, **e** antennae, and **f** lateral view of head

Fig. 5 Mortality kinetic for *M. domestica* adult [Bb-A] and larvae [Bb-L] after *B. bassiana* treatment, and larvae [Cc-L] after *C. citratus* application [reproduced from the data presented in Mishra and Malik (2012) and Kumar et al. (2013)]

larvae. However, the line representing larvae mortality due to *B. bassiana* infection had comparatively lesser slope to that for adult mortality. This could be explained due to absence of setae in larvae, which results in low infection, as explained earlier. However, as the mechanism of action of essential oil is different than that of fungal pathogens, with *C. citratus* oil setae plays no role in defining the extent of *M. domestica* mortality. Thus, the mortality of *M. domestica* larvae due to *C. citratus* was quite high from day 1 of application, as unlike fungal pathogens essential oils do not require any lag period for commencement of its action. Also, due to lack of self propagating nature, unlike fungal pathogens, mortality due to *C. citratus* remained low, and become constant after a certain time period. Similarly, the low infection

rate (10.1–20.2 %) in *M. domestica* pupae (Mishra and Malik 2013) may be attributed to their protective covering. Thus, the current study attests nature of fungal infection on outer surface of different life stages of *M. domestica*.

The field assay to validate *B. bassiana* infection under simulated condition against *M. domestica* larvae revealed 39.1 % mortality, whereas in the control treatment, 5.6 % larval mortality was observed. For the *M. domestica* pupae inoculated with *B. bassiana*, suppression of adult emergence was 16.7 %, while 75 % of emerged adult flies showed *B. bassiana* infection. The treated pupae also showed malformations in emerged adults. In the controlled assay, 3.3 % pupae showed suppression of adult emergence, while no infection was observed in the emerged adult flies. Although, the above assays confirms the *B. bassiana* efficacy in the field condition, their on-site practical use presents difficulty of application, stability, and economic viability (Mishra et al. 2011). Thus, the development of a suitable formulation becomes a crucial step towards successful implementation of *B. bassiana* in field condition. In this direction, previously our group prepared, characterized and tested the insecticidal activity of three different formulations of *B. bassiana* (Mishra et al. 2013). This study ascertained *B. bassiana* infection to different life stages of *M. domestica* through various field assays. Similarly, to overcome the barriers in actual application of essential oils, innovative formulations of essential oils have been used for verification of lab results during field investigations (Kumar et al. 2011, 2014). In the study by Kumar et al. (2011), emulsion formulation of essential oil was found to be good larvicidal and pupicidal agents against house flies, while efficacy of essential oil nanoparticle was depicted by Kumar et al. (2014). These studies validated the field applicability of the *B. bassiana* as well as efficacy of *C. citratus* against different life stages of *M. domestica* as depicted through microscopical investigation in the present study.

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Authors contribution Sapna Mishra contributed to this work by planning, executing and writing. Peeyush Kumar contributed to this work by planning, executing and writing. Anushree Malik contributed to this work by planning, writing and editing.

Compliance with ethical standards

Conflict of interest Authors' declare that the present research work do not have any kind of financial, academic, commercial, political or personal conflicts with any institution or individual.

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