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Effect of different concentrations of *Beauveria bassiana* on development and reproductive potential of *Spodoptera litura* (Fabricius)

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ABSTRACT

Beauveria bassiana, the most common and ubiquitous fungal entomopathogen is known to be highly potent for the control of insects belonging to various orders. The virulence of *B. bassiana* was tested against second, third and 4th instar larvae of *S. litura* using three concentrations i.e. 2.03×10^8, 4.03×10^6 and 1.47×10^5 spores/ml. All the treatments resulted in significantly higher mortality than control. Besides mortality, sublethal effects were also evaluated on larvae that survived fungal infection. Significant decrease in larval period was observed due to infection as compared to control. The life span of females emerging from treated larvae was half that of the control females. In addition to this, inhibitory effects were also manifested as reduced reproductive potential. The eggs descended from treated larvae showed significant decrease in hatchability. *B. bassiana* also induced pupal and adult deformities. A significantly higher number of deformed adults were observed at lower concentrations as compared to the highest concentration.

Key words: Entomopathogenic fungi, biological control, susceptibility, sublethal effects

INTRODUCTION

Spodoptera litura (Fabricius) (Lepidoptera: Noctuidae), commonly known as tobacco caterpillar, is one of the most destructive pest of cauliflower, groundnut, cotton, tomato, cabbage and other cruciferous crops (Anand *et al.*, 2009). It passes through 5-6 overlapping generations annually (Sasidharan and Varma, 2005; Kumar and Chapman, 2006) and if not controlled timely, it may result in huge crop losses ranging from 25.8-100 percent in various parts of India (Ahmad *et al.*, 2005). For the management of this pest, insecticide use is the most widely practiced. Although effective in reducing pest population in short term, these chemicals have little long term regulatory impact on pest population and often cause unwanted environmental side effects. The development of physiological resistance is one of the main reasons for this insect to become the key pest of many vegetable and field crops.

Biological control of insect pests using microorganisms is highly specific, of relatively low cost and low risk to ecosystem (Castillo *et al.*, 2000). Among these microorganisms, entomopathogenic fungi (EPF) play a significant role in controlling various crop pests. Unlike bacteria or viruses, EPF directly infect through insect cuticle and do not require ingestion for infection. Although 700 to 750 species of EPF have been reported as pathogenic to insects but only about a dozen have been exploited for insect control (Stark and Banks, 2003). Among these *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hyphocreales) is a facultative pathogen with wide host range (Armes *et al.*, 1997; Sahayaraj *et al.*, 2007). This fungus has potential to control over 70 insect pests belonging to different orders particularly lepidopteran pests, infesting various crops and appears to be innocuous to most non target organisms. Another important factor to be considered in favor of EPF is that, to date there has been no report of development of resistance. Effectiveness of control agents has been measured typically by percent mortality of treated population. However, these might affect various developmental stages, fecundity, longevity and reproductive potential of adults with potentially strong impacts on population growth and future abundance. Although sublethal effects of insecticides have been well documented by many workers but few references are available on EPF. Deleterious effects of the EPF *Aschersonia aleyrodis* (Deuteromycosina: Coelomycetes) were documented by Vargas *et al.* (1995) and Fransen (1987), when applied to *Trialeurodes vaporariorum* (Westwood). Changes in locomotion, excretion and food seeking behavior have also been observed in other species. Torrado - León *et al.* (2006) reported the sublethal effects of *B. bassiana* on *Bemisia tabaci* (Genadius). Reduced fecundity, preoviposition, oviposition and incubation period have been documented when ticks were treated with *Metarhizium anisopliae* (Metschnikoff) (Kaaya and Hassan, 2000). Such adverse effects on development of an insect, ultimately affect the population build up in next generation. In light of this, the present study was designed to evaluate the effect of various concentrations of *B. bassiana* on growth and development as well as reproductive potential of *S. litura* under laboratory conditions.

MATERIALS AND METHODS

Collection and rearing of insect

Larval stages of *S. litura* were collected from cauliflower fields adjoining Guru Nanak Dev University campus, Amritsar, Punjab (India) and maintained in battery jars (15 cm × 10 cm) at 25 ± 2°C and 60 - 70 percent relative humidity on cauliflower leaves. To avoid mortality due to unhygienic conditions the rearing jars were cleaned and fresh leaves were provided daily. The pupae were transferred in pupation jars having 2-3 cm layer of moist sterilized sand covered with filter paper. On emergence adults were shifted to oviposition jars lined with filter paper to facilitate egg laying. The adults were fed on 10% sugar solution. On hatching the larvae were shifted to artificial diet as recommended by Koul *et al.* (2004). Different instar larvae from this laboratory culture were used for various experiments.

Fungal source and preparation of spore suspension

The culture of *B. bassiana* (PDBC- Bb-5a) was procured from Project Directorate of Biological Control (PDBC), Bangalore, India. *B. bassiana* was cultivated and maintained on potato dextrose agar (PDA) medium. For conducting various experiments, 2-3 weeks old fungal culture was used. Conidia were harvested by scrapping the surface of culture with a sterile loop in 10 ml distilled water. A drop of 0.01 percent Tween 80 was added to it. The spore suspension was then filtered through muslin cloth to remove mycelia. Spore count was calculated using an improved Neubauer haemocytometer and the concentration was found to be 2.03×10^{8} spores/ml. The counts obtained for 10^{-1} and 10^{-2} dilutions were 4.03×10^{6} and 1.47×10^{5} spores/ml respectively. Spore viability was determined by plating 10 µl of the conidial suspension on PDA and percent germination of conidia was measured as described by Inglis *et al.*,1993.

Bioassays

Ten ml of each suspension were taken in a petri-dish and larvae were treated with each concentration using dipping method as indicated by Elizabeth Roy *et al.*, 2008. In case of control, larvae were treated with distilled water having a drop of 0.01 percent Tween 80. After air drying under laminar flow for 10-15 minutes the larvae were kept individually in the rearing tubes and allowed to feed on artificial diet. Virulence of *B. bassiana* was tested against second, third and 4th instar larvae. Each experiment was replicated 6 times with 20 larvae per replication. All experiments were repeated for confirmation of results. After treatment with different conidial concentrations, the larvae were incubated at 25 and 30°C and the humidity was maintained above 90 percent. Mortality was recorded daily. The dead larvae were surface sterilized by sodium hypochlorite and placed on petridish lined with moist filter paper. These petri-dishes were incubated at 25 ± 2°C to encourage fungal growth and sporulation in order to confirm infection of *B. bassiana*. *S. litura* larvae that showed mycelial growth were considered to have died of infection and only these counts were used to compute the pathogencity of *B. bassiana*. The slides were prepared by taking spores from dead larvae and observed under microscope to study its morphology.

Evaluation of sublethal effects

For this purpose a sublethal effect was considered to be any significant variation in development, longevity, fecundity or any type of deformity, relative to that measured in control. Larvae survived the fungal infection were reared on artificial diet till pupation at 25 ± 2°C and 60-70% relative humidity. Observations were made on larval period, percent pupation, adult emergence and any morphological deformity in various developmental stages.

The adults emerged from larvae survived fungal infections were observed to study for the effect of fungus on reproductive potential. The males and females were collected immediately after emergence and released in the oviposition jars in the ratio of 2:4. The experiment was replicated thrice. The adults were provided with 10% sugar solution and observations were made on adult longevity and fecundity. To study the effect of fungus on hatchability, the eggs laid in treatment and control were selected randomly with 1000 eggs per treatment. The experiment was replicated thrice and percent hatching was calculated.

Statistical analysis

Mortality was corrected by Abbott's formula (Abbott, 1925). The dose mortality response was evaluated by converting percentage mortality to probit mortality (Finney, 1971). The data were subjected to analysis of variance (ANOVA) followed by comparison of means of different treatments using least significant difference (LSD). Effect of temperature on larval mortality was analyzed using student's't' test. Correlation and regression equation was calculated between concentration and larval mortality. Analyses were performed using computer programming Minitab and SPSS – 10.

RESULTS

Larval mortality

Significantly higher mortality was achieved due to*B. bassiana* infection as compared to control. The highest percentage of dead larvae was recorded with concentration of 2.03 $\times 10^8$ spores/ml. Significant differences were recorded among the three concentrations of *B. bassiana* in second, third, and fourth instar larvae at 25°C. Similarly at 30°C all the treatments induced significantly higher mortality in second, third, and fourth instar larvae than that of control (Figure 1).

Data presented in figure 1 indicates significant effect of temperature on larval susceptibility. The spore count of 2.03×10^8 induced significantly higher mortality in second, third, and fourth larval instars at 30°C in comparison to 25°C temperature. The mean cumulative mortality of second instar larvae at 4.03 $\times 10^6$ and 1.47 $\times 10^5$ spores/ml also differed significantly at 25 and 30°C ($t = -4.47$, $df = 5$, $p = 0.007$; $t = -3.38$, $df = 5$, $p = 0.02$). However, except for highest concentration, the temperature did not have significant effect on mortality of fourth instar larvae. Rapid mortality rate was obtained at higher temperature, with the highest concentration inducing more than 35 % mortality within a week at 30°C as compared to less than 25 % at 25°C in all the larval instars. Similar trend in mortality distribution was observed at lower concentrations. Mortality was dose dependent. A significant positive correlation between concentration and mortality was observed at 25°C ($r^2 = 0.995$, $F = 375.80$, $p = 0.003$) and 30°C ($r^2 = 0.999$, $F = 1813.13$, $p = 0.001$) in second instar larvae. The correlation coefficients for third instar larvae were 0.966 ($F = 56.23$, $p = 0.017$) and 0.988 ($F = 163.39$, $p = 0.006$) at 25°C and 30°C respectively. Similar positive correlation was recorded for 4th instar larvae at 25°C ($r^2 = 0.967$, $F = 58.38$, $p = 0.017$) and 30°C ($r^2 = 0.967$, $F = 47.61$, $p = 0.020$). With the advancement of larval age, an increase in LC_{50} values was observed. For second instar larvae it was 5.00 $\times 10^4$ spores/ml which increased to 1.00 $\times 10^6$ and 5.01 $\times 10^6$ for third, and fourth instar at 30°C. However, at 25°C the LC_{50} values for second, third, and fourth instar larvae were 2.51 $\times 10^6$, 8.00 $\times 10^6$ and 1.26 $\times 10^7$ spores/ml, respectively.

Mycosis and sublethal effects

The infected larvae showed lesser movements. After death, the larvae became hard and stiff. At highest concentration mycelial growth on dead larvae started 1 day after death, but at lower concentrations it took 2 - 4 days to grow. The slides prepared from this fungal growth confirmed *B. bassiana* infection. In addition to larval mortality, *B. bassiana* also induced the following deleterious effects on larvae survived the fungal infection.

Although the fungal infection reduced the larval period than that of control but significant reduction was recorded in second and fourth instar treated larvae (Table 1). The differences were more evident at higher concentration than at lower concentrations. Adult emergence As is evident from figure 3, EPF adversely affected adult emergence from second, third, and fourth instar treated larvae as compared to control. Lesser number of adults (15.56 - 72.02%) emerged from fungus

Table 1. Effect of different concentrations of *B. bassiana* on Growth and developmental period of *S. litura*

Conc. (Spores/ml) / Instar	Larval Period (Days) (Mean±S.E)			Adult Emergence (%) (Mean±S.E)			Longevity (Days) (Mean±S.E)						Fecundity (Days) (Mean±S.E)			Hatching (%) (Mean±S.E)		
							Male			Female								
	second	third	4th	second	third	4th	second	third	4th	second	third	4th	second	third	4th	second	third	4th
2.03×10^8	15.86 ± 0.34	14.43 ± 0.39	12.31 ± 0.55	15.56 ± 10.42	60.00 ± 9.39	52.22 ± 9.45	7.00 ± 0.58	6.33 ± 0.33	6.33 ± 0.33	8.33 ± 0.60	8.00± 0.76	7.67 ± 0.67	202.00 ± 24.14	129.83 ± 5.04	235.20 ± 46.65	57.62± 11.08	43.33 ± 6.39	40.17 ± 2.74
4.03×10^6	16.48 ± 0.52	15.56 ± 0.43	12.51 ± 0.58	68.89 ± 7.78	67.89 ± 7.55	68.633 ± 9.83	6.67 ± 0.88	6.33 ± 0.33	7.33 ± 0.33	8.00 ± 0.58	7.33 ± 0.44	6.33 ± 0.88	714.63 ± 136.89	461.37 ± 162.28	636.70± 190.21	61.17 ± 5.78	57.13 ± 9.55	40.63 ± 5.82
1.47×10^5	17.11 ± 0.39	16.66 ± 0.40	13.93 ± 0.27	72.02 ± 7.43	69.40 ± 7.99	68.75 ± 8.44	5.33 ± 0.88	5.67 ± 0.67	6.00 ± 0.58	8.33 ± 1.59	6.63 ± 0.73	5.83 ± 0.44	725.00 ± 57.73	740.75 ± 61.05	795.50 ± 81.71	80.97 ± 3.11	69.44 ± 7.07	69.07 ± 2.88
Control	17.72 ± 0.48	16.68 ± 0.59	14.53 ± 0.35	77.38 ± 10.17	91.67 ± 4.01	92.78 ± 2.61	7.33 ± 0.88	6.67 ± 0.88	8.33 ± 0.33	10.17 ± 0.88	9.33 ± 0.67	11.17 ± 0.60	950.23 ± 42.40	859.70 ± 96.49	907.00 ± 83.36	90.57 ± 4.65	91.35 ± 5.25	91.83 ± 2.89
F value	N.S.	5.28*	6.99**	3.35*	4.98*	N.S.	N.S.	N.S.	5.71*	N.S.	N.S.	12.02**	12.34**	8.87*	6.97*	6.23*	42.02**	53.69**

** significant at 1%, * significant at 5%.

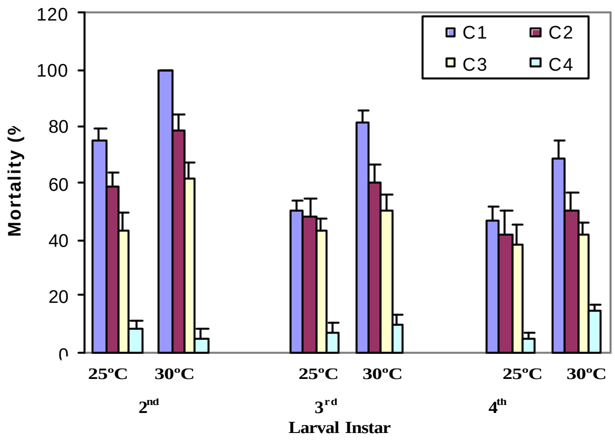


Figure 1. Effect of temperature on larval mortality of *S. litura* due to different conidial concentrations of *B. bassiana* (C1=2.03 × 10^8, C2=4.03 × 10^6 and C3=1.47 × 10^5 spores/ml, C4= control)

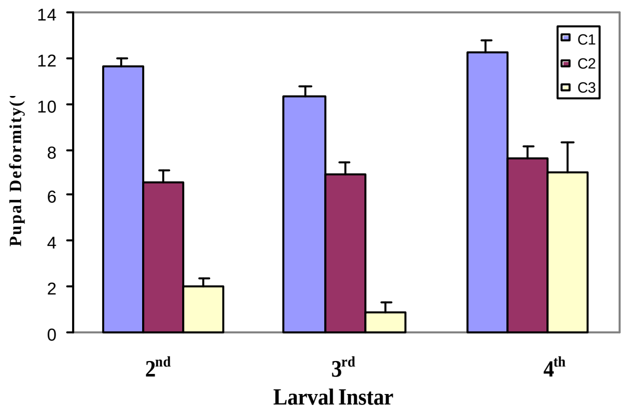


Figure 2. Pupal deformity due to larval treatment of *S. litura* with different concentrations of *B. Bassiana* (C1 = 2.03 × 10^8, C2 = 4.03 × 10^6 and C3 = 1.47 × 10^5 spores/ml).

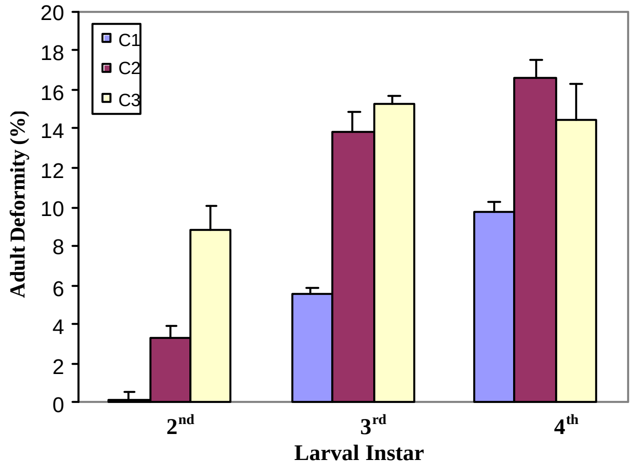
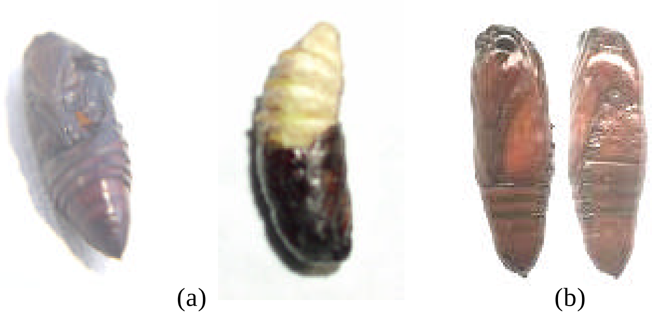


Figure 3. Adult deformity due to larval treatment of *S. litura* with different concentrations of *B. bassiana* (C1 = 2.03 × 10^8, C2 = 4.03 × 10^6 and C3 = 1.47 × 10^5 spores/ml)

(a) (b)

Figure 10. (a) Deformed adult (b) Normal adult



(a) (b)

Figure 8. (a) Deformed pupae, (b) normal pupae

treated larvae than control larvae (92.78 %). Adult emergence was significantly lesser at higher concentration than at lower concentrations with the least emergence from second instar larvae.

Adult longevity and fecundity

The secondary effects of fungal infection were also observed on adult longevity. Although the results were not significant except for 4^{th} instar larvae but life span of both the sexes reduced in all treatments as compared to control. The females emerged from control larvae lived longer as compared to treated larvae. The male longevity was also reduced by 1.00 - 2.33 days than that of control males (Table 1). Females emerged from treated larvae had significantly lower reproductive potential than those from control larvae. The effects were dose dependent i.e. with decrease in spore count fecundity increased. Significant differences were observed when treatment was given to second, third and fourth instar larvae (Table 1). The highest concentration was found to be most effective with average fecundity of 129.83 - 235.2 eggs/female, which was significantly lesser than control. As is evident from figure 6 hatching was significantly reduced in eggs descended from treatment of second, third, and fourth instar larvae. Viability of eggs was decreased from 90 percent in control to 40.17-80.97 percent in egg laid by adults emerged from treated larvae. The differences among the treatments were statistically significant and dose dependent (Table 1).

Deformities

Various kinds of deformities were observed due to fungal infection. During molting to pupae, the treated larvae failed to detach completely from the exuvium. Some pupae did not have fully formed cuticle. The second instar larvae suffered from 11.66, 6.61 and 2.08 percent pupal deformity when treated with 2.03×10^8, 4.03×10^6 and 1.47×10^5 spores/ml respectively. Similarly the pupal deformity was 6.94 - 10.33 and 7.01 - 12.26 percent respectively due to treatment of third, and fourth instar larvae (Figure 2). The adults emerged from the larvae that survived fungal infection also suffered from 3.33-16.67 percent deformities (Figure 3). However, no deformity was recorded in control. The deformed adults have crumpled and underdeveloped wings (Figure 10). Although the lower concentration induced more deformities as compared to highest concentration but the differences were non significant.

DISCUSSION

B. bassiana induced significant increase in larval mortality of *S. litura*. The concentration of 2.03×10^8 spores/ml was found to be very effective against all the larval stages. A significant positive correlation was recorded between concentration and mortality. Sasidharan and Varma (2005) also documented similar results for caterpillars of *Indarbela quadrinotata* Walker. The larvae suffered from 100 percent mortality when treated with *B. bassiana* @ 4×10^8 spores/ml whereas concentrations ranging from 2×10^6 to 2×10^8 spores/ml caused 66.7 % mortality within 10 days. However, when tested under field conditions efficacy of *B. bassiana* was reported to be reduced. Earlier Sandhu *et al*. (2001) reported 84 percent mortality in third instar larvae of *Helicoverpa armigera* (Hubner) when sprayed with *M. anisopliae* @ 1×10^5 spores/ml. Temperature is one of the important factor that influences susceptibility and development of disease in insects (Roberts and Campbell, 1997). During present investigations it was observed that concentration of 2.03×10^8 spore/ml exhibited 24.6% higher mortality in second instar larvae at 30°C than at 25°C. The LC_{50} value for all age group larvae were more at 25°C than at 30°C. As biochemical and physiological processes are strongly temperature dependent, thus temperature regulated activity of detoxification enzymes may influence insect susceptibility to various toxins (Toth and Sparks, 1990). Brattsten (1983) found that PSMO activity was higher in *Spodoptera eridantia* reared at 15ºC than larvae at 30ºC. Higher mortality observed at 30ºC may be linked with reduced enzymatic activity. Earlier

Liu *et al.* (1989) also obtained higher mortality in *Myzus persicae* (Sulzer) due to *B. bassiana* infection with the rise in temperature. But contrary to present findings Pandey and Kanaujia (2003) reported higher LC_{50} value at higher temperature while studying the effect of *B. bassiana* and *M. anisopliae* against *S. litura* at different temperatures. These differences may be due to variation in temperature requirement among fungal strain as suggested by Mc Coy *et al*. (1988).

The susceptibility of larvae decreased with the advancement of age. The early instar larvae experienced 28-32 % higher mortality than later instars at the highest concentration. Hafez *et al.* (1997) using *B. bassiana* against potato tuber moth, *Phthorimaea operculella* (Seller) showed higher susceptibility of 1^{st} and second instar larvae as compared to third and 4^{th} instars. Likewise Pandey and Kanaujia (2004) documented an increase of 1.96 and 3.02 times in LC_{50} value for third and 4^{th} instar respectively over second instar larvae of *S. litura* when treated with *M. anisopliae* at 25 ± 2°C. These differences in mortality among different instars may be related to enzymatic activity. It has been reported that the activity of detoxification enzymes varies considerably among and within developmental stages. The activity is low in egg stage, increases with each larval or nymphal instar and then declines to zero at pupation (Ahmad, 1986; Mullin, 1988)

In addition to increased mortality fungal infection also affected the growth and development of *S. litura*. Significant decrease in larval period was observed due to *B. bassiana* infection as compared to control. These results corroborate the findings of Batta and Abu-Safieh (2005) who reported decrease in life cycle of red flour beetle, *Tribolium castaneum* (Herbst) when treated with *M. anisopliae*. The negative effects of fungal infection on growth and development resulted in significant reduction in adult emergence, longevity as well as reproductive potential. The effects were dose dependent. Likewise, Hafez *et al.* (1997) documented decrease in emergence of *P. operculella*, from 100 % in control to zero percent at 16.5 × 10^8 conidia/ml. Decreased longevity of red palm weevil females due to fungal infection has also reported by Gindin *et al*. (2006). Reduction in longevity and fecundity of diamondback moth, *Plutella xylostella* (L.) due to insecticidal treatment has also been documented by Kumar and Chapman (2006).

Inhibitory effects such as reduction in fecundity and egg hatchability indicates that the fungus is invading the host. There is a possible link between sublethal infection and reproductive capacity of the adults as suggested by Mulock and Chandler (2001). However, fewer conclusive studies are available concerning sublethal effects of fungal pathogen on reproductive potential of an insect host. So it is hypothesized that the fungal infected larvae may have acquired and stored lesser nutrient resources than that of control larvae which might have affected the longevity and fecundity of females. In the insect the passage or carry over of nutrients from immature stages may provide proteins for oogenesis. Thus the fungal treatment of immature stages has a significant effect on mature stage. It may also be due to that the energy is diverted from biomass production to detoxification which ultimately resulted in reduced adult emergence and reproductive potential. Khachatourians (1986) also suggested that EPF caused the death of their host due to exhaustion of nutrients and liberation of toxins in the hemolymph. So, nutritional deficiency and toxins acting separately or in unison can drastically affect the development of an insect especially reproduction and molting which have high energetic demands. Earlier Sharma *et al.* (1994) reported physiological changes in *H. armigera* larvae after injecting them with filtrate of *B. bassiana* culture and found that the toxins destroy the normal balance of physiological system. The eggs descended from *B. bassiana* treated larvae showed significant reduction in hatchability. N'Doye (1976) also observed reduction in fertility of eggs laid by surviving *Chilo suppressalis* Walker when infected with *B. bassiana* as larvae. Similar reduction in egg viability of mosquitos infected with fungus, *Aspergillus parasiticus* Speare has been documented by Nnakumusana (1985). However, contrary to our results, Fargues *et al.* (1991) found that *B. bassiana* treatment had no significant effect on viability of eggs laid by surviving females of *Leptinotarsa decemlineata* (Say). Viability of eggs of western corn rootworm, *Diabrotica virgifera virgifera* LeConte was also not affected due to *B. bassiana* (Mulock and Chandler, 2001). However, Kaaya *et al.* (1996) reported reduction in fecundity and egg hatchability of *Rhipicephalus appendiculatus* and *Amblyomma variegatum* Fabricius following infection with *B. bassiana* and *M. anisopliae*.

In the present studies we observed pupal and adult deformities in *S. litura* due to fungal infection. Interference in the molting process has been documented by Torrado - León *et al.* (2006) in nymphs of *B. tabaci* when treated with *B. bassiana*. More than 30 % of the imagos resulting from treated nymphs were unable to detach completely from the exuvium. The present findings indicated that lower concentrations produced more sublethal effects as compared to higher concentration. Vargas *et al.* (1995) and Fransen (1987) also documented similar sublethal effects in *T. vaporariorum*, when was applied on *A. aleyrodis* before pupation. The fungus resulted in production of deformed adults. The larvae failed to molt properly into pupae. The molting process is highly dependent on nutrients for the formation of new cuticle. So, nutrient imbalance in the hemolymph due to fungal infection has the potential to interfere with any of the steps in this process.

The present findings conclude that EPF not only produce lethal effects but also adversely affect the various developmental stages of an insect. Reduction in longevity and fecundity ultimately affects population build up in the next generation. Although EPF have a wide application in integrated pest management strategies to reduce the load of chemical insecticides, but detailed studies need to be carried out on trophic interaction between crops, pests, natural enemies and EPF. Further to potentiate the efficacy of parasitoid and predators released after application of this pathogen, the behavioral changes like mobility, displacement and dispersal capacity of insect pests need to be determined at large scale. This is because the significantly reduced mobility and capacity to escape of a prey species could favor the functional response of predators and parasitoids and thus increasing their effectiveness as biological control agent.

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