

Vulnerability of Different Life Stages of *Bactrocera zonata* (Tephritidae: Diptera) Against Entomogenous Fungi

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Abstract.- The pathogenicity of three entomopathogenic fungi, *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea* was tested against different life stages of *Bactrocera zonata* under laboratory conditions using five different ways viz., contact bioassay, oral bioassay, inoculation of immatures and pupae and soil application for pupae. The results showed higher adult mortality in contact bioassay for all the fungi tested as compared to the other methods of application. The pathogenicity of *B. bassiana* was upto 20% in oral bioassay and 30-40% for *I. fumosorosea* and *M. anisopliae*, respectively. *I. fumosorosea* significantly affected the adult emergence, while 50-70% adult emergence was recorded in immatures inoculated with *B. bassiana* and *M. anisopliae*. Symptoms of dark, shrunk pupae, deformed adults and incomplete adult emergence from the pupae were also observed after immature treatments with insect pathogenic fungi.

Keywords: Insect pathogenic fungi, fruit fly, microbial control, adult mortality.

INTRODUCTION

Tephritid flies are considered a very destructive group of insect pests causing huge economic losses in agriculture, particularly in a variety of vegetables, fruits and flowers (Diamantidis *et al.*, 2008). Apple, guava, mango, musk melon and bitter melon are the major hosts of fruit flies recorded in Pakistan (Khan and Musakhel, 1999; Ahmad *et al.*, 2005). The rejection of fruits especially mangoes due to the presence of maggots is the greatest threat which makes it unfit for human consumption (Stonehouse *et al.*, 2002). Peach fruit fly, *Bactrocera zonata* (Saunders), melon fruit fly, *Bactrocera cucurbitae* Coquillett and oriental fruit fly, *Bactrocera dorsalis* Hendel are the three most notorious, destructive and widespread species of fruit flies in tropics. In Pakistan about eleven species of fruit flies have been recorded and most notable among them are *B. zonata*, *B. dorsalis*, *B. cucurbitae*, *Carpomyia incompleta*, *Carpomyia vesuviana*, *Myiopardalis pardalina*, *Dacus ferrugineus* and *Dacus diversus* (Abdullah and Latif, 2001; Abdullah *et al.*, 2002; Stonehouse *et al.*, 2002; Panhwar, 2005).

This prominent pest is a serious agriculture threat all over the world and stands for a danger to the successful establishment of horticulture industry and trade. It causes direct losses in the yield and marketability and in addition, the considerable threats posed by the pest are to the quarantine security and thus to international horticultural trade throughout the world (Joomaye *et al.*, 2000). The annual loss caused by the fruit flies in Pakistan is estimated to be 200 million dollars at farm level, which is in turn increasing the extensive use of cover insecticide sprays (Abbasi *et al.*, 1992; Mahmood *et al.*, 1995; Saika and Dutta, 1997; Singh *et al.*, 2000; Ali *et al.*, 2010).

Various groups of insecticides viz., organophosphates, carbamates, synthetic pyrethroids and new chemistry are in indiscriminate use by the farmers as cover sprays (Stonehouse *et al.*, 1997; Alston, 2002; Ahmad *et al.*, 2005; El-Aw *et al.*, 2008). The insecticides of various natures like dipterex, triazophos, imidacloprid, and neem products are in notable use for the management of fruit flies. The sole method to control insect pests in fruits and vegetables is to use insecticides that cause environmental pollution and hygienic problems, which also represent a risk for human and wild life (Gallo, 2007). The enormous use of synthetic pesticides has led to the development of insecticide resistant pest species in the last four decades (Casida and Quistad, 1998). Numerous problems have

* Corresponding author: sfareed@bzu.edu.pk
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occurred due to the continuous use of insecticide for fruit fly control by different methods of application, either the soil application for larvae or newly emerged adult control or foliage application for adult control. These setbacks have provoked the need for searching biological control alternatives, including microbes (Toledo, 2002). In an integrated management program for fruit flies and other important agricultural insect pests, the microbial control can replace other methods to some extent especially the use of agrochemicals, presenting economic and environmental advantages for the tropics (Oliveira *et al.*, 2010; Bahar *et al.*, 2011; Freed *et al.*, 2012a, b; Akmal *et al.*, 2013; Khan *et al.*, 2014).

Various isolates of *Metarhizium anisopliae* (Metschnikoff) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycotina: Hyphomycetes) have proved to be effective against adults and pupae of *Ceratitis capitata* under laboratory and greenhouse conditions (Lacey *et al.*, 2001; Ekesi *et al.*, 2002, 2005; Dimbi *et al.*, 2003; Konstantopoulou and Mazomenos, 2005; Quesada *et al.*, 2006; Almeida *et al.*, 2007). Entomogenous fungus, *Isaria fumosorosea* (Wize) Brown and Smith (Deuteromycotina: Hyphomycetes) has been reported to reduce fecundity and fertility of the Mediterranean fruit fly (Castillo *et al.*, 2000). The application of conidia to soil has revealed to be an effective mode to infect newly emerged adults (Lezama *et al.*, 2000; Dimbi *et al.*, 2003; Ekesi *et al.*, 2005). For biological control of fruit flies, some commercial products are currently available, which are based on different strains of entomopathogenic fungi (Lacey *et al.*, 2001).

The objective of the study under this background was to evaluate the effectiveness of *B. bassiana*, *I. fumosorosea* and *M. anisopliae* as biological control agent when used against different life stages of *B. zonata* with different methods of application; in addition the evaluation of the best application method through bioassays was also the part of this study.

MATERIALS AND METHODS

Insects

The fruit flies, *B. zonata* were collected from

infected mango fruits for this experiment. The culture of *B. zonata* was established in the Laboratory of Insect Microbiology and Biotechnology, Department of Entomology, Bahauddin Zakariya University, Multan, Pakistan at 27±1°C temperature and 65±5% relative humidity (RH). The adults were maintained in transparent plastic rearing cages of size 2×2×3 ft³. Fresh water and adult diet consisted of sugar: yeast = (3:1) was provided on daily basis.

Fungal cultivation

The isolates of *B. bassiana* (BB-02, isolated from *Rhyzopertha dominica*), *M. anisopliae* (MA-02, isolated from soil of cotton field), and *I. fumosorosea* (IF-02, isolated from soil of vegetable field), were morphologically identified using standard taxonomic keys. For this purpose the slants of single conidial cultures of the isolates grown on PDA were used. For further propagation the spores from these slants were spread onto the PDA plates (9 cm diameter) and these plates were kept at 25°C in darkness at 70-75% RH for 14 days. After required growth fungal spores were harvested to treat the insects or stored at 4°C until used for insect bioassay.

Bioassays

Contact bioassay

Twenty-five newly emerged adults were positioned in separate cages with provision of adult diet and water on daily basis. Three different concentrations 1×10⁸, 2×10⁸, 3×10⁸ spores/ml of all the three entomopathogenic fungi were prepared in Tween 80 (0.05%) solution by haemocytometer and adults were then sprayed cautiously for one minute with a small sprayer (Mahmoud, 2009). The same numbers of adults were treated in control, but sprayed only with sterile Tween 80 (0.05%) solution and diet was provided under similar conditions as described above. The experiment confined of five replications for each treatment, while dead adults were counted for consecutive seven days to calculate the percent mortality.

Oral bioassay

Suspensions of *B. bassiana*, *M. anisopliae* and *I. fumosorosea* were prepared and mixed with

the diet for adults (twenty five adults per cage). The treated diet was consumed by the flies within 2-3 days. In control, diet containing Tween 80 (0.05%) solution was used instead of conidial spores. The experiment with different concentrations of all fungi was replicated five times and mortality was recorded continuously for seven days.

Inoculation of immatures

B. zonata last instar maggots, which were near to pupate within next twenty four hours were used for the experiment. Each treatment was replicated five times with twenty five maggots in each treatment. Immatures were sprayed with the fungi for upto one minute with different concentrations of fungi, while the same number of insects in control were sprayed with sterile Tween 80 (0.05%) solution and were later on observed for normal pupation, fungal effects and adult emergence. The experiment was conducted under similar temperature and humidity conditions as described earlier.

Inoculation of pupae

Twenty five fresh pupae were taken from the culture and inoculated by spraying with three different concentrations of all fungi for one minute. Five replications of each treatment were used under the same temperature and humidity conditions as described previously. Same numbers of pupae were used for control and were sprayed with sterile Tween 80 (0.05%) solution. All the pupae were then observed for fungal effect and adult emergence.

Soil application for pupae

All the three concentrations of insect pathogenic fungi were used to treat the soil. Each standard concentration (5ml) was used to treat 50g lots of sterile soil. Fresh pupae were then placed in the treated soil till adult emergence to check the fungal effect and pupal mortality. Each treated soil contained twenty five pupae with five replications each. The pupae used for control were placed in soil sprayed with sterile Tween 80 (0.05%) solution, with the same number of pupae used for each treatment, while the laboratory conditions were

maintained same as described earlier.

Statistical analysis

Completely Randomized Design was used for the experiments of all fungi with four treatments including control and each treatment was replicated five times. Tukey's HSD Test was used to separate the means for the corrected mortality. For each comparison the $P = 0.05$ level of significance was used. All statistical analysis was performed by using SAS (SAS, 2002), while to calculate the LC_{50} and LT_{50} values, probit analysis was used (Finney, 1971).

RESULTS

Effect on adults in contact bioassay

The insect pathogenic fungi, *B. bassiana* showed significant mortality from the 1st day post treatment which was more than 25% with the concentration of 2×10^8 , but 100% mortality was obtained on the 6th day after treatment with the same concentration of 2×10^8 ($F_{4,6} = 8.42$; $P < 0.0001$), minimum mortality observed was upto 90% in *B. bassiana* with concentration of 1×10^8 ($F_{4,6} = 11.32$; $P < 0.0001$) (Fig.1a). The LC_{50} values, 3.13×10^8 spores/ml (4.24×10^5 - 2.31×10^8) (Table I) showed that 50% mortality was obtained at 2nd day of treatment, while LT_{50} values were in range of 2.86-3.39 days at different concentrations of *B. bassiana*, respectively (Table II). The treatment of *M. anisopliae* also showed noteworthy results in contact bioassay. Significant mortality was observed from the 2nd day after treatment in all tested concentrations however 100% mortality was observed in all concentrations, 1×10^8 ($F_{4,6} = 9.90$; $P < 0.0001$), 2×10^8 ($F_{4,6} = 8.88$; $P < 0.0001$), 3×10^8 ($F_{4,6} = 14.74$; $P < 0.0001$) (Fig.1b) after 7th days. The LC_{50} values, 1.68×10^8 spores/ml (1.17×10^8 - 2.40×10^8) (Table I) portrayed that the 50% mortality was obtained at 4th day of treatment, while LT_{50} values were in range of 3.66- 3.76 days at different concentrations of *M. anisopliae*, respectively (Table II). *I. fumosorosea* showed significant mortality from the 1st day post treatment but maximum mortality was observed on the 6th day after treatment at a concentration of 3×10^8 ($F_{4,6} = 26.25$; $P < 0.0001$) and the minimum mortality on the 7th day after

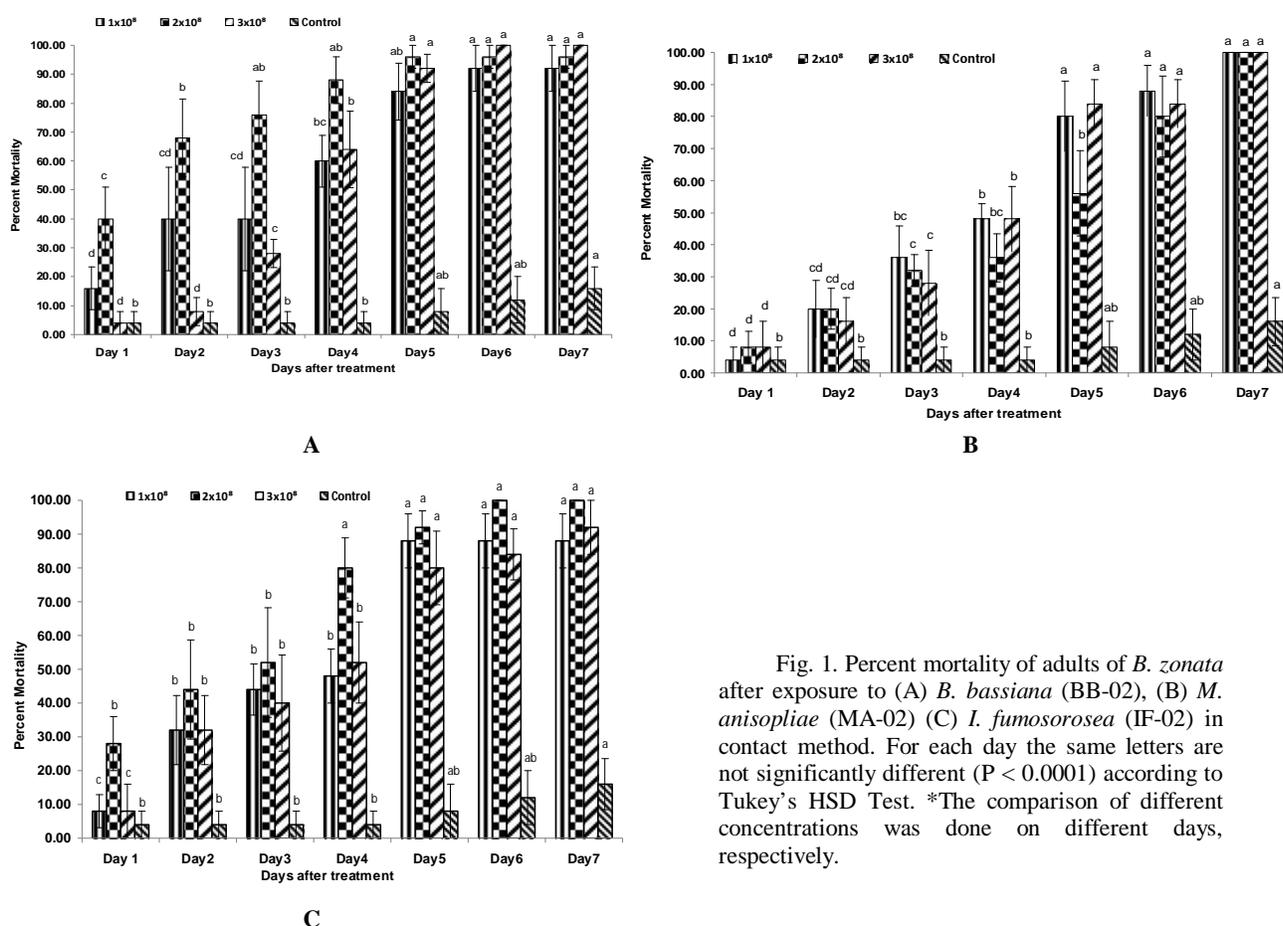


Fig. 1. Percent mortality of adults of *B. zonata* after exposure to (A) *B. bassiana* (BB-02), (B) *M. anisopliae* (MA-02) (C) *I. fumosorosea* (IF-02) in contact method. For each day the same letters are not significantly different ($P < 0.0001$) according to Tukey's HSD Test. *The comparison of different concentrations was done on different days, respectively.

Table I.- LC₅₀ (spores/ml) of entomopathogenic fungi in spray bioassay on different days against *B. zonata* adults.

Fungi	Day	LC ₅₀	FD Limit	Slope	DF
<i>B. bassiana</i>	2 nd	3.13×10^8	$(4.24 \times 10^5 - 2.31 \times 10^8)$	0.77 ± 1.67	1
<i>I. fumosorosea</i>	4 th	1.68×10^8	$(1.17 \times 10^8 - 2.40 \times 10^8)$	1.89 ± 0.76	1
<i>M. anisopliae</i>	4 th	2.46×10^8	$(8.80 \times 10^7 - 6.88 \times 10^8)$	0.77 ± 0.74	1

treatment was observed in 1×10^8 ($F_{4,6} = 18.70$; $P < 0.0001$) (Fig.1b). The LC₅₀ values, 2.46×10^8 spores/ml ($8.80 \times 10^7 - 6.88 \times 10^8$) (Table I) showed that 50% mortality was obtained at 4th day of treatment, while LT₅₀ values were in range of 4.19-3.97 days at different concentrations of *I. fumosorosea*, respectively (Table II). In contrast to all these, less than 20% mortality was recorded in

control (Fig.1c).The results showed all fungal isolates to be effective against the adults of *B. zonata* but *B. bassiana* showed effective control in short period.

Table II.- LT₅₀ (days) of different concentrations of entomopathogenic fungi in spray bioassay against *B. zonata* adults.

Fungi	Conc.	LT ₅₀	FD Limit	Slope	DF
<i>B. bassiana</i>	1×10^8	3.39	(2.99-3.85)	4.24 ± 0.59	5
	2×10^8	1.62	(1.27-2.08)	2.81 ± 0.44	5
	3×10^8	2.86	(2.51-3.25)	4.58 ± 0.60	5
<i>I. fumosorosea</i>	1×10^8	3.66	(3.24-4.14)	4.31 ± 0.61	5
	2×10^8	2.49	(2.14-2.91)	3.74 ± 0.50	5
	3×10^8	3.76	(3.33-4.24)	4.32 ± 0.62	5
<i>M. anisopliae</i>	1×10^8	3.97	(3.63-4.35)	6.53 ± 0.89	5
	2×10^8	4.54	(4.14-4.98)	6.00 ± 0.88	5
	3×10^8	4.19	(1.05-16.73)	7.99 ± 4.66	5

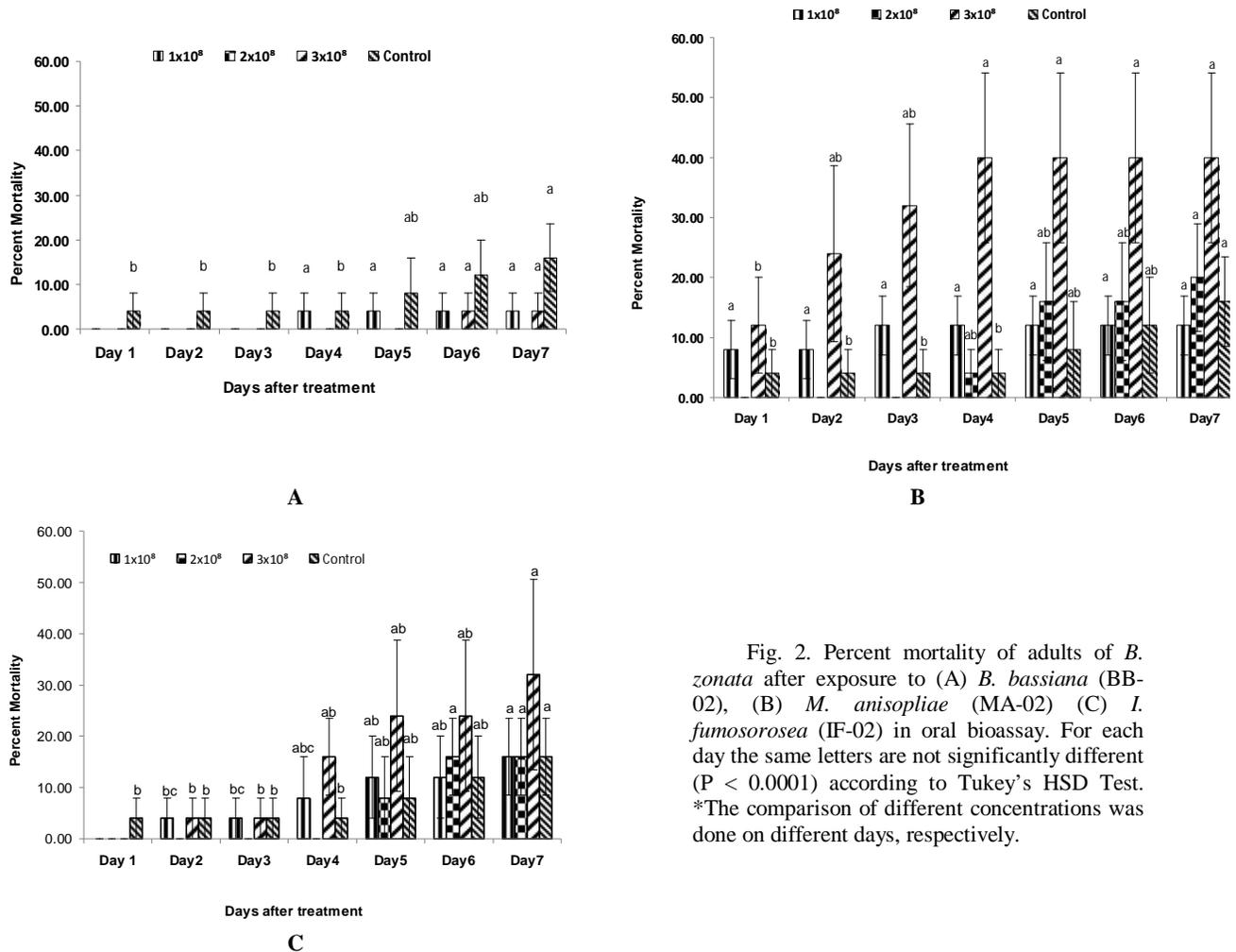


Fig. 2. Percent mortality of adults of *B. zonata* after exposure to (A) *B. bassiana* (BB-02), (B) *M. anisopliae* (MA-02) (C) *I. fumosorosea* (IF-02) in oral bioassay. For each day the same letters are not significantly different ($P < 0.0001$) according to Tukey's HSD Test. *The comparison of different concentrations was done on different days, respectively.

Effect on adults in oral bioassay

In *B. bassiana* treatment, no significant mortality was recorded in all three concentrations of entomopathogenic fungus. Only less than 10% mortality was recorded in the concentrations of 1×10^8 and 3×10^8 (Fig. 2a). In the experiment with *M. anisopliae*, 3×10^8 showed some mortality percentage from the first day post treatment and the maximum mortality recorded was 40% on the 4th day after treatment with 3×10^8 ($F_{4,6} = 19.57$; $P < 0.0001$) (Fig. 2b). *I. fumosorosea* showed maximum mortality of upto 30% on the 7th day post treatment at a concentration of 3×10^8 ($F_{4,6} = 3.66$; $P = 0.0044$), as compared to the control, while on the same day, 1×10^8 and 2×10^8 showed the same mortality results as that of control (Fig. 2c). The

current findings proved this method to be ineffective against *B. zonata* as it showed less than 50% mortality.

Effect on larvae and adult emergence in larval inoculation method

In larval inoculation method of *B. zonata* with *B. bassiana*, the significant adult emergence (70%) was recorded on 7th day post treatment at 1×10^8 ($F_{4,6} = 19.40$; $P < 0.0001$) (Fig. 3a). In *M. anisopliae*, maximum 50% adult emergence was recorded on 4th day after treatment with 2×10^8 ($F_{4,6} = 17.75$; $P < 0.0001$) (Fig. 3b). No significant adult emergence occurred in the larval inoculation method with *I. fumosorosea*. Very low emergence was observed in 2×10^8 ($F_{4,6} = 1.00$; $P = 0.4706$) (Fig. 3c)

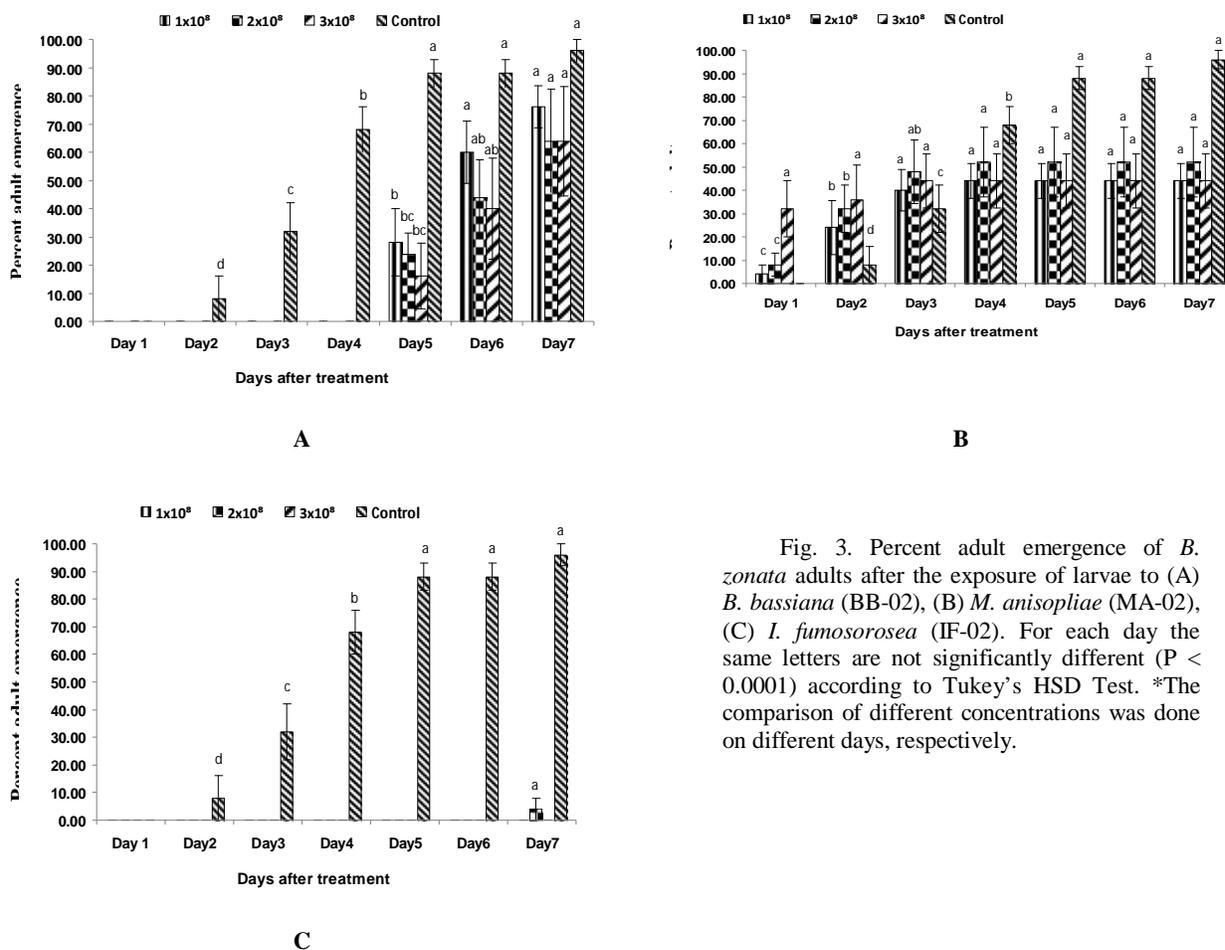


Fig. 3. Percent adult emergence of *B. zonata* adults after the exposure of larvae to (A) *B. bassiana* (BB-02), (B) *M. anisopliae* (MA-02), (C) *I. fumosorosea* (IF-02). For each day the same letters are not significantly different ($P < 0.0001$) according to Tukey's HSD Test. *The comparison of different concentrations was done on different days, respectively.

as compared to control, which proved *I. fumosorosea* to be more effective than the other fungi tested against the larval stage of *B. zonata*.

Effect on pupae and adult emergence in pupae inoculation method

Significant adult emergence of more than 80% was recorded from *B. bassiana* treated pupae on the 2nd day, at 2×10^8 ($F_{4,6} = 7.35$; $P < 0.0001$) (Fig. 4a) as compared to control, while 1×10^8 and 3×10^8 showed maximum adult emergence of less than 80% on the 4th and 7th day, respectively (Fig. 4a). In the *M. anisopliae* treated pupae, 90% adult emergence was recorded on 5th day at the concentration of 1×10^8 ($F_{4,6} = 32.67$; $P < 0.0001$), while, 80% at 2×10^8 ($F_{4,6} = 23.96$; $P < 0.0001$) and 3×10^8 ($F_{4,6} = 17.77$; $P < 0.0001$) (Fig. 4b), respectively on 4th day after adult emergence started

as compared to the control. The adult emergence in *I. fumosorosea* treated pupae on 7th day recorded 80-90% adult emergence in all the three concentrations of this entomopathogenic fungus *i.e.*, 1×10^8 ($F_{4,6} = 22.37$; $P < 0.0001$), 2×10^8 ($F_{4,6} = 23.85$; $P < 0.0001$), 3×10^8 ($F_{4,6} = 32.29$; $P < 0.0001$) (Fig. 4c) as compared to the control. The results showed that all three tested fungi had no significant effect on the pupal mortality of *B. zonata*.

Effect on pupae and adult emergence in soil application method

In soil application method, the adult emergence was recorded earlier with application of 1×10^8 spores/ml of *B. bassiana*. Maximum adult emergence was 85-90% in all the three concentrations 1×10^8 ($F_{4,6} = 21.93$; $P < 0.0001$), 2×10^8 ($F_{4,6} = 18.82$; $P < 0.0001$), 3×10^8 ($F_{4,6} =$

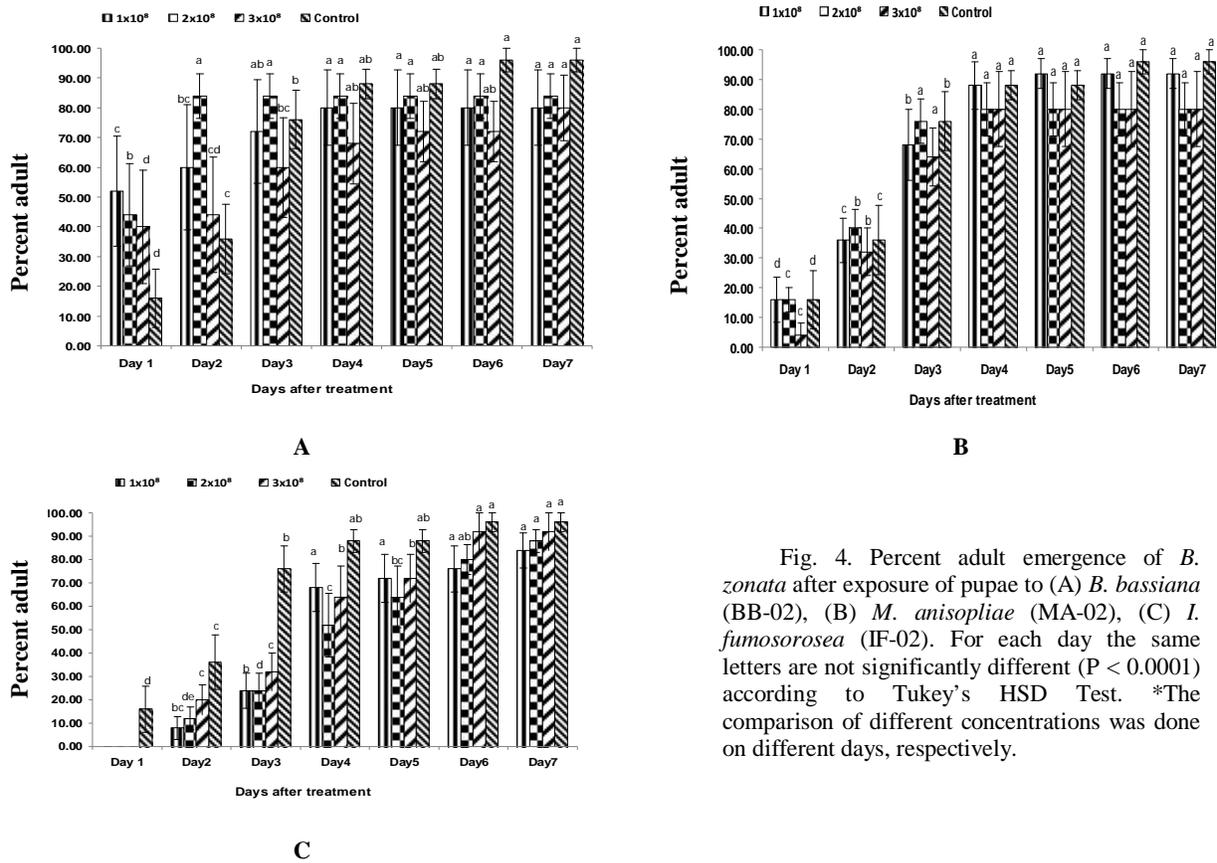


Fig. 4. Percent adult emergence of *B. zonata* after exposure of pupae to (A) *B. bassiana* (BB-02), (B) *M. anisopliae* (MA-02), (C) *I. fumosorosea* (IF-02). For each day the same letters are not significantly different ($P < 0.0001$) according to Tukey's HSD Test. *The comparison of different concentrations was done on different days, respectively.

24.96; $P < 0.0001$) (Fig.5a) as compared to control. In the *M. anisopliae* treated soil, significant adult emergence was recorded viz., 1×10^8 ($F_{4,6} = 8.60$; $P < 0.0001$) showed maximum adult emergence of more than 95% and the other concentrations i.e., 2×10^8 ($F_{4,6} = 13.99$; $P < 0.0001$), 3×10^8 ($F_{4,6} = 8.28$; $P < 0.0001$) (Fig. 5b) showed > 80% adult emergence as compared to control. In *I. fumosorosea* treated soil, adult emergence from the pupae was recorded from 60-90% in all the three concentrations of 1×10^8 ($F_{4,6} = 10.00$; $P < 0.0001$), 2×10^8 ($F_{4,6} = 41.91$; $P < 0.0001$), 3×10^8 ($F_{4,6} = 100.03$; $P < 0.0001$) (Fig. 5c) as compared to control. The results showed that all three tested fungi had no significant effect on the pupal mortality of the fruit fly.

DISCUSSION

The virulence of three entomopathogenic

fungi, *B. bassiana*, *I. fumosorosea* and *M. anisopliae* was checked for the control of *B. zonata*. The vulnerability of adult tephritid flies to entomopathogenic fungi has been confirmed by result reported by different authors (Carswell *et al.*, 1998; Dimbi *et al.*, 2003; Ekesi *et al.*, 2005; Yee and Lacey, 2005; Bahar *et al.*, 2011). The mortality results of our experiments are within the ranges that have been already reported for different fungal species and strains for fruit flies. In the treatment with *B. bassiana*, Dimbi *et al.* (2003) found 7 to 100% mortality in adults of *Ceratitis rosa* var. *fasciventris* and *Ceratitis capitata*. In the evaluation studies of 16 strains of *B. bassiana* against *C. capitata* adults, Munoz (2000) recorded mortality level from 20 to 98.7%. In the same way, Campos (2000) reported a mortality range between 82 and 100% in the evaluation tests of *B. bassiana* and *M. anisopliae* for the control of *Anastrepha ludens* adults.

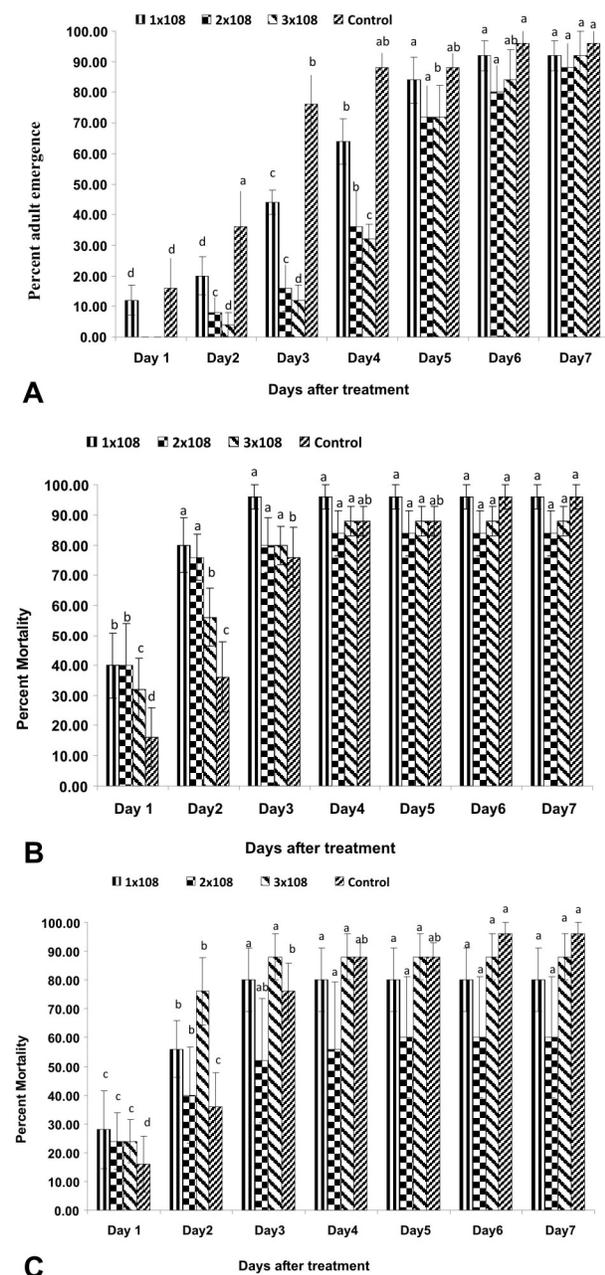


Fig. 5. Percent adult emergence of *B. zonata* adults after the exposure of soil and pupae to (A) *B. bassiana* (BB-02), (B) *M. anisopliae* (MA-02), (C) *I. fumosorosea* (IF-02). For each day the same letters are not significantly different ($P < 0.0001$) according to Tukey's HSD Test. *The comparison of different concentrations was done on different days, respectively.

Moderate pathogenicity of *B. bassiana* has also been reported by Konstantopoulou and Mazomenos (2005) when evaluated as oral bioassay against *Bactrocera oleae* adults. The results coincide with the findings of our studies where pathogenicity of *B. bassiana* was upto 20% in oral bioassay and upto 30-40% for *I. fumosorosea* and *M. anisopliae*, respectively. Application of insect pathogenic fungi to the soil is a practicable fruit fly management substitute in coffee groves and orchards (Garcia *et al.*, 1989). In current studies this application method was most successful in case of *M. anisopliae*, as in spite of adult emergence, more than 50% adults failed to eclose properly (Fig. 6e). Different types of morphological changes were observed in the larvae and pupae treated with entomopathogenic fungi. Some immatures treated with *B. bassiana* failed to emerge after pupation. In addition the pupae became dark with black ends or brown or dark brown colored markings were found on the whole pupa with dark end points (Fig. 6b).

The most distinguished character which was observed in the pupae formed from the *M. anisopliae* treated last instars, in which the pupae were discolored and the adults failed to emerge properly. Only the adult head emerged but rest of the body failed to come out of pupae. The findings illustrate that the adults emerged from the *M. anisopliae* treated immature or pupae were more in female ratio and also found with deformed abdomen. Deformed nymphs were observed in *Schistocerca gregaria* when treated with *M. anisopliae*. Nymphs were unable to get out from old cuticle, in addition discoloration, deformation at wings, tibia and abdomen was also reported (Elbanna *et al.*, 2012). Morphological abnormalities after treatment of *M. domestica* with entomopathogenic fungi include small, shrunken larvae, distorted pupae and failure of adult emergence (Khater, 2003).

According to the findings of the current study, all tested entomopathogenic fungi proved to be successful for the management of fruit fly at larval and adults stages especially in the contact bioassay.

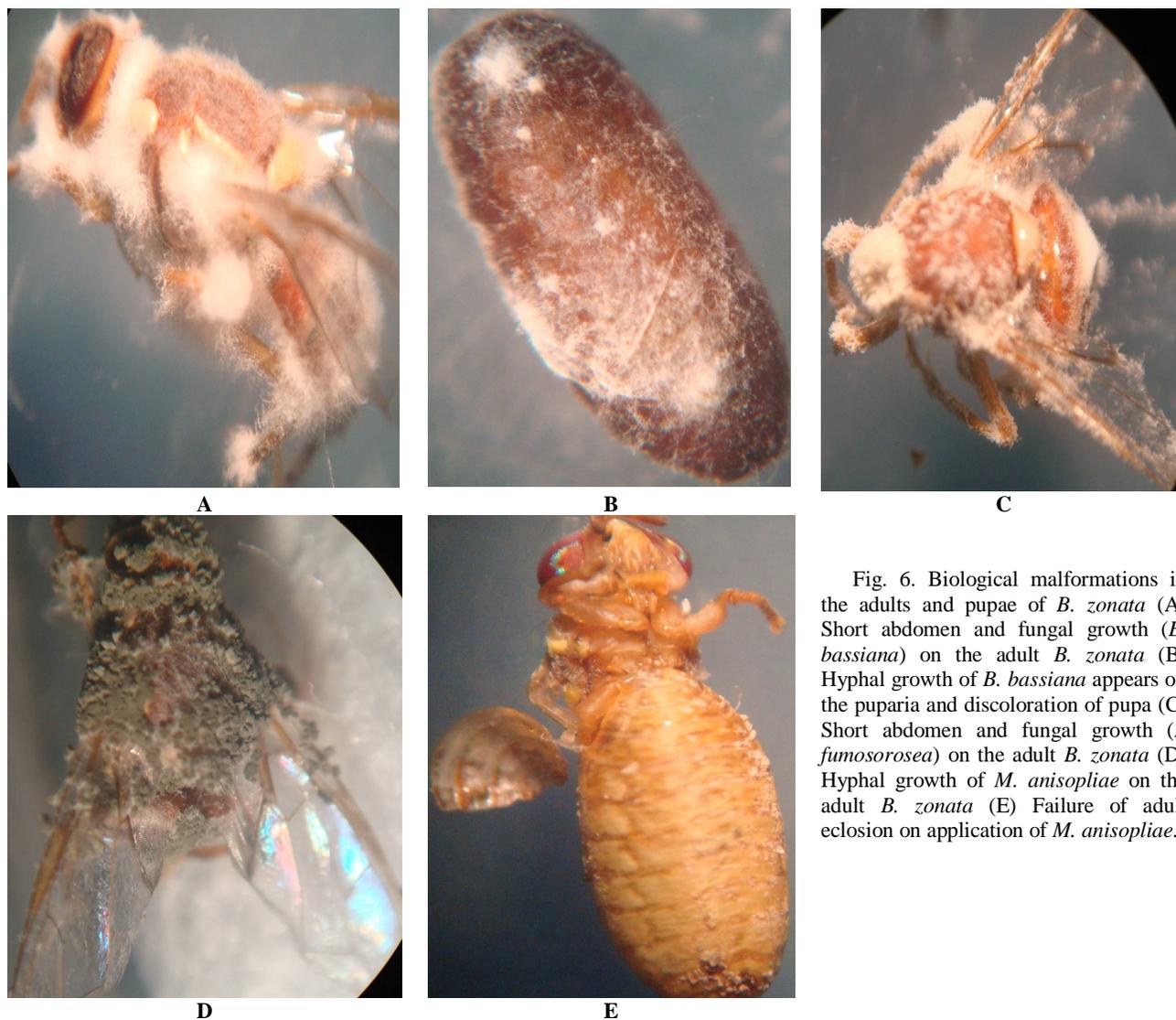


Fig. 6. Biological malformations in the adults and pupae of *B. zonata* (A) Short abdomen and fungal growth (*B. bassiana*) on the adult *B. zonata* (B) Hyphal growth of *B. bassiana* appears on the puparia and discoloration of pupa (C) Short abdomen and fungal growth (*I. fumosorosea*) on the adult *B. zonata* (D) Hyphal growth of *M. anisopliae* on the adult *B. zonata* (E) Failure of adult eclosion on application of *M. anisopliae*.

ACKNOWLEDGEMENT

We are grateful to Bahauddin Zakariya University, Multan for funding this research.

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(Received 6 September 2013, revised 17 January 2014)

