Phytoparasitica (2013) 41:221–234
DOI 10.1007/s12600-012-0282-9

Effects of interactions among *Metarhizium anisopliae*, *Bacillus thuringiensis* and chlorantraniliprole on the mortality and pupation of six geographically distinct *Helicoverpa armigera* field populations

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Received: 16 June 2011 / Accepted: 10 December 2012 / Published online: 19 January 2013

Abstract A local isolate of *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae), *Bacillus thuringiensis* subsp. *kurstaki* and chlorantraniliprole were assessed against six field populations of tomato fruitworm *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) in a series of laboratory bioassays. Two dose rates of *B. thuringiensis* (0.5, 1 μg g^{-1}), one of both *M. anisopliae* (1.3×10^{6} conidia ml^{-1}) and chlorantraniliprole (0.01 ppm) were applied alone and in combination with each other against 2nd, 3rd, 4th and 5th larval instars. The mortality was observed every 24 h until pupation. The bioassays were carried out at 25°C and 75% r.h. The highest mortality was observed in Rawalpindi with the lowest pupation rate by applying the combined concentrations of *B. thuringiensis* and chlorantraniliprole. The lowest mortality was observed in population from Gujranwala among all the tested populations. The antagonistic interaction was noted where the high dose rate of *B. thuringiensis* was combined with *M. anisopliae*; however, the remaining interactions enhanced the mortality and reduced the percent pupation. The overall results demonstrated that all the treatments gave significant control of the larval instars of *H. armigera*. The population from Gujranwala proved least susceptible whereas the one from Rawalpindi was highly susceptible.

Keywords Entomopathogenic fungi · Bacteria · Fruitworm · Anthranilic diamide insecticide · Populations · Tomato

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Introduction

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae) is a cosmopolitan polyphagous insect pest of economically important crops (Cherry *et al.* 2000; Wakil *et al.* 2009a; b; 2010). Synthetic insecticides continue to be the main controlling agents but several cases of resistance and reduced susceptibility of *H. armigera* to insecticides and environmental and

human health concerns have been reported worldwide (Gunning *et al.* 1998; Martin *et al.* 2000; Qaim *et al.* 2008). The injudicious and repeated use of insecticides resulted in the development of resistance in *H. armigera* populations in different localities of Punjab province, Pakistan (Ahmad *et al.* 2001; 2003). This situation prompted the researchers to test the safe alternatives like *Metarhizium anisopliae*, *Bacillus thuringiensis* and a new chemical insecticide against geographically distinct populations. Similarly, Purwar & Sachan (2006) emphasized eco-friendly alternatives because they have been showing good results for the protection of agricultural crops.

Among the alternatives, entomopathogenic fungi are getting serious attention due to their environmental safety and pest selectivity (Carner & Yearian 1989). The efficacy of entomopathogenic fungi is well documented by Nguyen *et al.* (2007), who reported promising results of seven strains of *Metarhizium anisopliae*, *Beauveria bassiana* and *Paecilomyces fumosoroseus* against different larval stages of *H. armigera*. The fungal spores germinate and penetrate the cuticle by making germ tubes and proliferate in the hemolymph, which later produce new propagules (Zimmermann 2007).

Bacillus thuringiensis Berliner is a spore-forming gram positive bacterium which is considered as an effective insecticide harmless to natural enemies, quite safe to mammals and environmentally acceptable (Entwistle *et al.* 1993). *B. thuringiensis* toxins bind to specific receptors located on the brush border membrane of midgut columnar cells, which eventually leads to cell death (Bravo *et al.* 2004).

Chlorantraniliprole (Coragen®) powered by Rynaxypyr is a reduced risk new class of chemistry, the anthranilic diamides, which has an excellent environmental profile due to low mammalian toxicity and low residual effect. It works through ingestion, contact, ovicidal and ovi-larvicidal activity (Lahm *et al.* 2007). The muscle contraction is controlled by managing the balance of calcium levels in the muscle cells through ryanodine receptors. The chlorantraniliprole makes the ryanodine receptors open and release all the stored calcium, which causes the death of insects by the rapid cessation of feeding, lethargy, regurgitation and muscle paralysis (Cordova *et al.* 2007).

Considering the significance of these promising alternatives and the paucity of data on the interaction of chlorantraniliprole with microbial agents, the present study was designed to evaluate the separate and combined effects of *M. anisopliae*, *B. thuringiensis* and chlorantraniliprole on the mortality of 2nd, 3rd, 4th and 5th larval instars of *H. armigera* collected from different geographical localities of Punjab province, Pakistan; the pupation rate of the larvae was also assessed.

Materials and methods

Rearing of study insects Six populations of *H. armigera* were collected from Gujranwala, Sheikhupura, Faisalabad, Lahore, Sargodha and Rawalpindi districts of Punjab, Pakistan, and reared on artificial diet in the IPM Laboratory, Department of Agricultural Entomology, University of Agriculture, Faisalabad. These larvae were reared in 32-well plastic trays (6 cm in diameter × 5.5 cm in depth) until pupation. The adults were kept in plastic jars (15 cm in diameter × 19 cm in depth) lined with coarse tissue paper as nappy liner for egg laying. They were provided with 10% honey solution in a 5-ml test tube plugged with cotton and placed vertically on the top of the jar. The eggs were surface sterilized with 0.5% sodium hypochlorite followed by two changes of distilled water and were placed in plastic bags for hatching (Marzban *et al.* 2009). The newly emerged larvae were fed on artificial diet (Wakil *et al.* 2011) at 25± 2°C, 70±5% r.h. synchronized at a photoperiod of 14:10 (L:D) hours.

Chlorantraniliprole It is a novel insecticide in the anthranilic diamide class powered by Rynaxypyr which is a semi-viscous liquid off-white in color. It contains (20% w v^{-1}) chlorantraniliprole (200 ml l^{-1}) and (80% w v^{-1}) other ingredients (800 ml l^{-1}) provided by DuPont™ Operations Private Limited, Pakistan.

Bacillus thuringiensis toxin The wettable powder (WP) commercial formulation (Dipel) containing *B. thuringiensis* subspecies *kurstaki* with a density of active toxin 3.2%, other inert material 96.8% with the potency of 16,000 i.u., was provided by BioSciences Corporation (Libertyville, IL, USA). One gram of powder was dissolved in 2 ml of sterile distilled water and gently streaked on the nutrient agar media (5 g peptone, 5 g NaCl, 1.5 g beef extract, 1.5 g

yeast extract, 15 g agar and 1,000 ml distilled H_2O) added with suitable antibiotic. Then, the spores and crystals were collected by centrifugation at 16,000 rpm for 15 min at 4°C temperature for the extraction of *Bt* toxin (Crecchio & Stotzky 2001; Hernández *et al.* 2005). The pellet was washed three times with cold 1 M NaCl and re-suspended in 1 M NaCl. Estimation of spore-crystal concentration was carried out in 1:100 dilutions by measuring the optical density at 600 nm (Hernández *et al.* 2005) and the samples were stored in the refrigerator until used.

Fungal isolation and conidial preparation *Metarhizium anisopliae* isolate was originally isolated from the soil sample collected from harvested tomato fields in the Rawalpindi district (Pakistan). The fungus was isolated using the Galleria bait method (Zimmermann 1986) with third or fourth larval instars of the wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae). The larvae before baiting were immersed in water at 56°C for 15 s in order to minimize their ability to produce silk webbing in the soil (Woodring & Kaya 1988). The soil sample was sieved through 5 mm mesh and 60 g (Rodrigues *et al.* 2005) of soil was poured in the plastic cups (6 cm high, 4.5 cm diam). Ten larvae of *G. mellonella* were placed in cups sealed with the perforated lids and incubated at 25°C. The cups were shaken and inverted daily for the first 5 days to ensure the movement of the larvae in the soil. After 15–20 days the dead cadavers were shifted to other cups and surface sterilized with 0.05% sodium hypochlorite. The cups were provided with the moist filter paper and incubated at 25°C until the appearance of external growth of fungi. The fungi were identified morphologically by preparing the slides and the fungus was sub-cultured on Sabouraud Dextrose Agar (32.5 g SDA; 7.5 g of Bacto Agar; 5 g yeast in 1 *l* distilled water) for mass production and incubated at 25°C, 75% r.h. with 16 h illumination per day. After 14 days of incubation, the plates were kept under the aluminum foil roasting pan on the bench top for 1 week for drying. The fungal conidia were harvested by scraping the plates using a sterilized (70% ethanol) scalpel. The fungal conidia were dissolved in 0.05% Tween-80 solution and filtered through muslin cloth to remove the mycelial debris. The desired concentrations were recorded by dilution plate count method (Marannino *et al.* 2006), estimating the colony forming units.

Mortality and pupation of H. armigera larval instars Two concentrations of *B. thuringiensis* (0.5 and 1 μg g^{-1}), one of *M. anisopliae* (1.3×10^6 conidia ml^{-1}) and chlorantraniliprole (0.01 ppm) individually and 0.5 μg g^{-1} of *B. thuringiensis* + *M. anisopliae*, 1 μg g^{-1} of *B. thuringiensis* + *M. anisopliae*, 0.5 μg g^{-1} of *B. thuringiensis* + chlorantraniliprole and 1 μg g^{-1} of *B. thuringiensis* + chlorantraniliprole were applied to assess the mortality and pupation of different larval (L2–L5) instars of *H. armigera*. The larvae in vials without any treatment served as control. *B. thuringiensis* and chlorantraniliprole were applied by mixing in artificial diets. So, five batches of artificial diets were prepared: two batches having two different concentrations of *B. thuringiensis* (0.5 and 1 μg g^{-1}), one batch of chlorantraniliprole (0.01 ppm), one batch for 0.5 μg g^{-1} of *B. thuringiensis* + chlorantraniliprole and one batch for 1 μg g^{-1} of *B. thuringiensis* + chlorantraniliprole. The treatments were thoroughly mixed in an electric shaker for 30 s in 1 *l* jug to distribute them evenly in the artificial diets. Then the pre-starved (24 h) larval instars of each population were put separately in the plastic vials (base radius 2.8 cm × height 7 cm) and allowed to feed separately on each treated batch of artificial diet (1 cm^3 piece) for 48 h. Then the fed larvae were removed and immersed individually for 10 s into fungal solution. The treated larvae were allowed to crawl freely in an empty petri dish to remove an excess of fungal suspension and were put in plastic vials containing an artificial diet until the larvae died or pupated. The bioassays were conducted at 25±2°C, 75% r.h. and L16:D8 h photoperiod. Each treatment consisted of 20 larvae for every population and the bioassays were repeated three times independently to avoid the phenomenon of pseudo-replication. The data for mortality were recorded after every 24 h and the last count was recorded after 12 days for all the populations and larval instars (L2–L5). After removing the dead individuals, the remaining larvae were kept until pupation. The larvae were prodded with a blunt needle and those unable to move in a coordinated manner were considered as dead (Ma *et al.* 2008).

Statistical analysis The data were transformed with arcsine square root to check the homogeneity and the normality of error variances before analysis. The data were analyzed by analysis of variance with Minitab 13.2 (Minitab, 2002 Software Inc., Northampton, MA,

Table 1 Mean mortality and pupation (% ± SE) of second instar *H. armigera* larvae from six field populations treated with *B. thuringiensis* (Bt1, Bt2: 0.5 and 1 μg g^{-1}), *M. anisopliae* (Ma: 1.3×10^{6} conidia ml^{-1}) and Chlorantraniliprole (Ch: 0.01 ppm) individually and in combination. (Means sharing the same letter within each population do not differ significantly; HSD at 5% significance)

Populations	Treatments	Actual mortality	Pupation	Expected mortality	Co-toxicity factor	Type of interaction
Gujranwala	Bt2+Ch	83.76±2.35a	11.27±2.07f	67.64	23.83	Synergistic
	Bt1+Ma	63.85±1.09b	30.90±1.09e	49.73	28.40	Synergistic
	Bt1+Ch	61.05±2.15b	33.70±2.15e	52.69	15.86	Additive
	Bt2+Ma	50.41±0.61c	44.34±0.61d	64.68	−22.06	Antagonistic
	Ch	36.76±3.29d	57.97±3.27c			
	Ma	33.80±2.16d	60.95±2.16c			
	Bt2	30.87±1.32d	63.88±1.32c			
	Bt1	15.93±1.98e	78.82±1.98b			
	Control	2.38±0.97f	95.82±1.79a			
Sheikhupura	Bt2+Ch	87.82±0.03a	6.94±0.04f	70.85	23.94	Synergistic
	Bt1+Ma	68.94±2.95b	25.82±2.96e	53.94	27.81	Synergistic
	Bt1+Ch	66.04±2.64b	28.72±2.64e	56.94	15.98	Additive
	Bt2+Ma	52.57±3.73c	42.19±3.73d	67.85	−22.53	Antagonistic
	Ch	38.17±1.35d	56.59±1.35c			
	Ma	35.17±2.77d	59.60±2.77c			
	Bt2	32.68±1.78d	62.08±1.78c			
	Bt1	18.77±2.24e	76.06±2.22b			
	Control	2.32±2.32f	96.27±0.92a			
Faisalabad	Bt2+Ch	92.50±2.20a	3.14±1.55f	73.22	26.33	Synergistic
	Bt1+Ma	74.04±3.89b	20.73±3.89e	57.12	29.63	Synergistic
	Bt1+Ch	70.59±2.09b	24.18±2.09e	60.84	16.02	Additive
	Bt2+Ma	53.71±2.73c	41.06±2.73d	69.50	−22.71	Antagonistic
	Ch	40.02±2.38d	54.75±2.38c			
	Ma	36.30±1.76d	58.47±1.76c			
	Bt2	33.20±1.60d	61.57±1.60c			
	Bt1	20.82±1.99e	73.95±1.99b			
	Control	2.59±0.74f	95.78±1.21a			
Lahore	Bt2+Ch	100.00±0.00a	0.00±0.00f	78.93	26.70	Synergistic
	Bt1+Ma	77.76±3.81b	16.06±3.46e	62.81	23.80	Synergistic
	Bt1+Ch	76.87±1.99b	17.97±1.99e	64.19	19.75	Additive
	Bt2+Ma	57.32±2.03c	37.45±2.03d	77.55	−26.09	Antagonistic
	Ch	41.84±2.28d	52.93±2.28c			
	Ma	40.46±1.98d	54.31±1.98c			
	Bt2	37.09±1.97d	57.68±1.97c			
	Bt1	22.35±2.08e	72.42±2.08b			
	Control	1.93±1.01f	97.07±1.01a			
Sargodha	Bt2+Ch	100.00±0.00a	0.00±0.00f	81.55	22.63	Synergistic
	Bt1+Ma	82.40±3.62b	10.34±2.07e	66.77	23.42	Synergistic
	Bt1+Ch	81.77±1.97b	12.97±1.97e	68.33	19.67	Additive
	Bt2+Ma	58.47±2.63c	36.27±2.63d	79.99	−26.90	Antagonistic
	Ch	43.39±1.65d	50.65±1.34c			
	Ma	41.83±2.14d	52.91±21.4c			

Table 1 (continued)

Populations	Treatments	Actual mortality	Pupation	Expected mortality	Co-toxicity factor	Type of interaction
	Bt2	38.16±1.83d	56.58±1.83c			
	Bt1	24.94±1.25e	69.80±1.25b			
	Control	2.73±0.77f	94.89±1.25a			
Rawalpindi	Bt2+Ch	100.00±0.00a	0.00±0.00e	82.88	20.66	Synergistic
	Bt1+Ma	85.75±3.61b	8.81±3.61e	70.18	22.19	Synergistic
	Bt1+Ch	84.29±2.14b	10.27±2.14e	71.42	18.02	Additive
	Bt2+Ma	61.11±2.82c	33.45±2.82d	81.63	−25.14	Antagonistic
	Ch	44.18±0.94d	48.85±2.06c			
	Ma	42.93±2.53d	50.89±2.54c			
	Bt2	38.70±2.36d	55.86±2.36c			
	Bt1	27.24±1.45e	67.32±1.45b			
	Control	2.02±1.04f	96.41±0.96a			

USA) with significance detected at $P=0.05$. Means for mortality and pupation were separated and compared with Tukey's Kramer test (HSD) (Sokal & Rohlf 1995). The type of interaction between different treatments was determined by equation CTF = (*Oc*-*Oe*)/*Oe* × 100, where CTF is the co-toxicity factor, *Oc* is the observed percentage mortality resulted from the combined application, and *Oe* the expected percentage mortality, that is, the total percentage produced by each of the treatments used in the combination (Mansour *et al.* 1966). The interactions were categorized into three groups: a positive factor of 20 or more meaning synergism, a negative factor of 20 or more meaning antagonism, and any intermediate value (*i.e.*, between −20 and +20) was considered additive (Mansour *et al.* 1966; Wakil *et al.* 2012).

Results

There were significant differences in mortality in all the tested populations when treated with the combined or individual concentrations of *M. anisopliae*, *B. thuringiensis* and chlorantraniliprole. The main effects were (localities: $F_{5,647}=171.44$, $P\leq0.01$; larval instars: $F_{3,647}=1372.73$, $P\leq0.01$; treatments: $F_{8,647}=2648.04$, $P\leq0.01$) and their associated interactions (localities x larval instars: $F_{15,647}=4.62$, $P\leq0.01$; localities × treatments: $F_{40,647}=9.29$, $P\leq0.01$; larval instars x treatments: $F_{24,647}=72.47$, $P\leq0.01$). The synergistic effects on the mortality of *H. armigera* larval instars were exhibited by the combined applications of low dose of *B. thuringiensis* with *M. anisopliae* and high dose of *B. thuringiensis* with chlorantraniliprole. The additive interaction was evident when a low dose of *B. thuringiensis* and chlorantraniliprole was combined; however, a high dose of *B. thuringiensis* showed antagonistic interaction with *M. anisopliae* (Table 1). The highest mortality (100%) of 2nd instar larvae of *H. armigera* was observed in Rawalpindi ($F_{8,26}=173$, $P\leq0.01$), Sargodha ($F_{8,26}=270$, $P\leq0.01$) and Lahore ($F_{8,26}=178$, $P\leq0.01$) populations; the lowest pupation recorded was in Rawalpindi ($F_{8,26}=176$, $P\leq0.01$) followed by Sargodha ($F_{8,26}=308$, $P\leq0.01$) and Lahore ($F_{8,26}=223$, $P\leq0.01$) by applying the combination of *B. thuringiensis* (1 μg g^{-1}) with chlorantraniliprole; however, the lowest mortality ($F_{8,26}=152$, $P\leq0.01$) was observed in Gujranwala, with ($F_{8,26}=132$, $P\leq0.01$) pupation.

The combined treatments of chlorantraniliprole with a high dose of *B. thuringiensis* showed higher mortality (100%) of the 3rd instar larvae from Rawalpindi ($F_{8,26}=391$, $P\leq0.01$) and Sargodha ($F_{8,26}=565$, $P\leq0.01$) with minimum pupation (Rawalpindi: $F_{8,26}=291$, $P\leq0.01$; Sargodha: $F_{8,26}=480$, $P\leq0.01$), compared with control treatment (Table 2). The lowest mortality ($F_{8,26}=77.4$, $P\leq0.01$) and pupation ($F_{8,26}=115$, $P\leq0.01$) was observed in Gujranwala. The larval mortality was significantly increased in combined rather than individual treatments. The interaction between the combined treatments was synergistic and additive; however, the

Table 2 Mean mortality and pupation (% ± SE) of third instar *H. armigera* larvae from six field populations treated with *B. thuringiensis* (Bt1, Bt2: 0.5 and 1 μg g^{-1}), *M. anisopliae* (Ma: 1.3×10^{6} conidia ml^{-1}) and Chlorantraniliprole (Ch: 0.01 ppm) individually and in combination (Means sharing a common letter within each population do not differ significantly; HSD at 5% significance)

Populations	Treatments	Actual mortality	Pupation	Expected mortality	Co-toxicity factor	Type of interaction
Gujranwala	Bt2+Ch	81.33±3.13a	13.26±3.13f	64.58	25.94	Synergistic
	Bt1+Ma	60.76±2.63b	33.83±2.63e	47.35	28.32	Synergistic
	Bt1+Ch	58.28±2.05bc	36.31±2.05de	49.63	17.43	Additive
	Bt2+Ma	48.80±0.90c	47.11±1.78d	62.3	−21.67	Antagonistic
	Ch	34.89±2.74d	60.29±2.70c			
	Ma	32.61±2.16d	63.31±2.89c			
	Bt2	29.68±1.32d	66.10±1.92c			
	Bt1	14.74±1.98e	79.85±1.98b			
	Control	2.74±1.40f	97.29±0.74a			
Sheikhupura	Bt2+Ch	83.34±3.03a	11.40±3.03f	66.16	25.96	Synergistic
	Bt1+Ma	63.04±2.71b	31.70±2.71e	49.41	27.6	Synergistic
	Bt1+Ch	60.55±2.62bc	34.19±2.62de	52.31	15.75	Additive
	Bt2+Ma	49.60±2.42c	45.14±2.42d	63.26	−21.59	Antagonistic
	Ch	36.03±2.27d	58.71±2.27c			
	Ma	33.12±2.92d	61.62±2.92c			
	Bt2	30.13±2.10d	64.61±2.10c			
	Bt1	16.28±1.83e	78.46±1.83b			
	Control	3.36±1.07f	95.64±1.07a			
Faisalabad	Bt2+Ch	85.33±2.65a	9.38±2.56f	69.01	23.65	Synergistic
	Bt1+Ma	67.27±2.18b	27.44±2.18e	52.31	28.61	Synergistic
	Bt1+Ch	64.37±2.12b	30.34±2.12e	55.72	15.53	Additive
	Bt2+Ma	51.84±1.64c	42.87±1.64d	65.6	−20.98	Antagonistic
	Ch	37.32±0.97d	57.39±0.96c			
	Ma	33.91±2.35d	60.80±2.35c			
	Bt2	31.69±0.72d	63.02±0.72c			
	Bt1	18.39±2.02e	76.32±2.02b			
	Control	2.65±0.92f	97.44±0.93a			
Lahore	Bt2+Ch	87.68±2.90a	7.03±2.90f	70.86	23.74	Synergistic
	Bt1+Ma	70.38±3.62b	24.33±3.62e	55.3	27.28	Synergistic
	Bt1+Ch	67.80±2.40b	26.91±2.40e	58.45	16.00	Additive
	Bt2+Ma	52.55±2.70c	38.82±1.78d	67.71	−22.39	Antagonistic
	Ch	38.33±0.65d	56.38±0.65c			
	Ma	35.18±2.26d	59.53±2.26c			
	Bt2	32.53±2.17d	62.18±2.17c			
	Bt1	20.11±1.63e	74.60±1.63b			
	Control	3.09±0.89f	95.93±1.12a			
Sargodha	Bt2+Ch	100.00±0.00a	0.00±0.00f	79.97	25.05	Synergistic
	Bt1+Ma	83.14±0.19b	11.53±0.19e	65.13	27.65	Synergistic
	Bt1+Ch	78.62±1.37b	16.05±1.37e	67.63	16.25	Additive
	Bt2+Ma	57.32±2.03c	37.35±2.03d	77.47	−26.01	Antagonistic
	Ch	42.92±2.59d	51.75±2.59c			
	Ma	40.42±1.57d	54.25±1.57c			

Table 2 (continued)

Populations	Treatments	Actual mortality	Pupation	Expected mortality	Co-toxicity factor	Type of interaction
	Bt2	37.05±1.60d	57.62±1.60c			
	Bt1	24.71±0.83e	69.96±1.01b			
	Control	3.76±1.27f	94.91±1.28a			
Rawalpindi	Bt2+Ch	100.00±0.00a	0.00±0.00f	81.99	21.96	Synergistic
	Bt1+Ma	84.29±0.95b	10.42±0.95e	67.36	25.14	Synergistic
	Bt1+Ch	80.84±2.34b	13.87±3.14e	69.58	16.19	Additive
	Bt2+Ma	58.43±0.96c	36.28±0.97d	79.77	−26.75	Antagonistic
	Ch	43.79±1.54d	50.92±1.54c			
	Ma	41.57±0.96d	53.14±0.96c			
	Bt2	38.20±0.97d	56.51±0.97c			
	Bt1	25.79±2.72e	68.92±2.72b			
	Control	4.08±1.02f	94.14±1.48a			

antagonistic interaction was noted with combined application of 1 μg g^{-1} of *B. thuringiensis* + *M. anisoplaie* showing −21.67 co-toxicity factor.

The mortality of 4th instar larvae of *H. armigera* was again higher, with less pupation, when exposed to the combined treatments of *B. thuringiensis* (1 μg g^{-1}) with chlorantraniliprole (Table 3). The larval mortality was significantly increased in the population from Rawalpindi ($F_{8,26}$=101, P≤0.01) and with pupation ($F_{8,26}$=94.2, P≤0.01). The Gujranwala population was less susceptible ($F_{8,26}$=41.2, P≤0.01), followed by Sheikhupura ($F_{8,26}$=80.4, P≤0.01), Faisalabad ($F_{8,26}$=110, P≤0.01) and Lahore ($F_{8,26}$=121, P≤0.01) and the same trend was exhibited from L2–L5 of *H. armigera*. The synergistic and additive interaction was observed in all combined treatments except high dose of *B. thuringiensis* with *M. anisopliae* treatment was antagonistic in all the tested populations. Among individual treatments the chlorantraniliprole showed significantly more mortality in all populations tested with maximum 35.51% and 59.12% pupation in Rawalpindi; and least in Gujranwala with 24.67% mortality and 69.96% pupation.

The synergistic and additive effect was noted in combined treatments against 5th instar larvae of *H. armigera* among all the populations tested and mortality was higher in the combined than individual treatments (Table 4). The antagonistic interaction was noted in the high dose of *B. thuringiensis* with *M. anisopliae* treatment. The high dose of *B. thuringiensis* with chlorantraniliprole application against various populations showed the decreasing mortality trend (Rawalpindi: $F_{8,26}$=55.9, P≤0.01; Sargodha: $F_{8,26}$=44.2, P≤0.01; Lahore: $F_{8,26}$=59.1, P≤0.01; Faisalabad: $F_{8,26}$=55, P≤0.01; Sheikhupura: $F_{8,26}$=59.5, P≤0.01; Gujranwala: $F_{8,26}$=30.6, P≤0.01); however, the pupation tendency was in ascending order (Rawalpindi: $F_{8,26}$=113, P≤0.01; Sargodha: $F_{8,26}$=55.7, P≤0.01; Lahore: $F_{8,26}$=71, P≤0.01; Faisalabad: $F_{8,26}$=55.1, P≤0.01; Sheikhupura: $F_{8,26}$=66, P≤0.01; Gujranwala: $F_{8,26}$=49.4, P≤0.01).

Discussion

The present studies were conducted to determine the influence of individual and combined applications of *M. anisopliae, B. thuringiensis* and chlorantraniliprole against different larval instars of field populations of *H. armigera*. The entomopathogenic fungi have great potential to control lepidopterous insect pests (Vega-Aquino *et al.* 2010), confirming the present study in which *M. anisopliae* showed satisfactory results against different larval instars. Laboratory bioassays demonstrating the effectiveness of *M. anisopliae* against the various larval instars of *H. armigera* (Nguyen *et al.* 2007) gave further confirmation of the present findings. Several isolates of *M. anisopliae* have also shown high levels of virulence against the various forest pests (Remadevi *et al.* 2010); similarly, 90% mortality of both *Agriotes obscurus* L. (Coleoptera: Elateridae) and the unidentified species

Table 3 Mean mortality and pupation (% ± SE) of fourth instar *H. armigera* larvae from six field populations treated with *B. thuringiensis* (Bt1, Bt2: 0.5 and 1 μg g^{-1}), *M. anisopliae* (Ma: 1.3×10^{6} conidia ml^{-1}) and Chlorantraniliprole (Ch: 0.01 ppm) individually and in combination (Means sharing a common letter within each population do not differ significantly; HSD at 5% significance)

Populations	Treatments	Actual mortality	Pupation	Expected mortality	Co-toxicity factor	Type of interaction
Gujranwala	Bt2+Ch	53.91±2.69a	40.72±2.69f	43.44	24.09	Synergistic
	Bt1+Ma	44.80±3.63ab	49.83±3.63ef	32.41	38.22	Synergistic
	Bt1+Ch	39.59±3.56bc	55.04±3.56de	36.99	7.01	Additive
	Bt2+Ma	30.34±1.96cd	64.29±1.96cd	38.86	−21.94	Antagonistic
	Ch	24.67±1.95de	69.96±1.95bc			
	Ma	20.09±3.11de	74.54±3.11bc			
	Bt2	18.78±2.56de	75.85±2.56bc			
	Bt1	12.33±0.95ef	82.30±0.95b			
	Control	3.45±1.19f	95.55±1.19a			
Sheikhupura	Bt2+Ch	61.36±1.46a	33.42±1.46f	49.24	24.61	Synergistic
	Bt1+Ma	52.77±2.08ab	42.13±1.98ef	38.93	35.52	Synergistic
	Bt1+Ch	47.63±1.19b	47.15±1.19e	43.52	9.43	Additive
	Bt2+Ma	34.85±2.60c	59.93±2.60d	44.66	−21.95	Antagonistic
	Ch	28.11±1.95cd	66.67±1.95cd			
	Ma	23.52±3.14de	71.26±3.14bc			
	Bt2	21.13±2.65de	73.65±2.65bc			
	Bt1	15.41±1.53e	79.37±1.55b			
	Control	4.34±0.57f	94.15±0.37a			
Faisalabad	Bt2+Ch	66.72±3.75a	28.10±3.75f	53.91	23.76	Synergistic
	Bt1+Ma	58.38±2.51ab	36.44±2.51ef	42.64	36.89	Synergistic
	Bt1+Ch	51.69±0.96b	43.13±0.96e	48.31	6.98	Additive
	Bt2+Ma	36.96±1.94c	57.86±1.94d	48.24	−23.38	Antagonistic
	Ch	30.34±0.34cd	64.48±0.34cd			
	Ma	24.67±1.99de	70.15±1.99bc			
	Bt2	23.56±1.72de	71.26±1.72bc			
	Bt1	17.97±1.026e	76.85±1.02b			
	Control	3.04±0.92f	96.44±0.89a			
Lahore	Bt2+Ch	70.80±2.08a	23.89±2.08f	56.94	24.35	Synergistic
	Bt1+Ma	61.81±2.88ab	32.88±2.89ef	45.35	36.30	Synergistic
	Bt1+Ch	55.65±2.68b	39.04±2.68d	51.35	8.38	Additive
	Bt2+Ma	38.52±2.89c	56.17±2.89cd	50.94	−24.38	Antagonistic
	Ch	32.23±1.73cd	62.46±1.73cd			
	Ma	26.23±1.55de	68.47±1.55bc			
	Bt2	24.71±1.01de	69.98±1.01bc			
	Bt1	19.12±1.24e	75.57±1.24b			
	Control	3.48±0.98f	94.83±1.33a			
Sargodha	Bt2+Ch	74.40±2.89a	20.41±2.89e	59.96	24.07	Synergistic
	Bt1+Ma	66.25±1.57ab	28.56±2.57de	49.64	33.46	Synergistic
	Bt1+Ch	60.97±2.12b	33.84±2.92d	54.49	11.87	Additive
	Bt2+Ma	42.88±3.12c	52.93±2.88c	55.11	−22.19	Antagonistic
	Ch	33.30±1.89cd	61.51±1.89bc			
	Ma	28.44±3.12d	66.37±3.12b			

Table 3 (continued)

Populations	Treatments	Actual mortality	Pupation	Expected mortality	Co-toxicity factor	Type of interaction
	Bt2	26.67±2.94d	68.14±2.94b			
	Bt1	21.20±2.82d	73.61±2.82b			
	Control	2.95±1.18e	97.75±0.64a			
Rawalpindi	Bt2+Ch	77.45±2.63a	17.18±2.63f	62.83	23.26	Synergistic
	Bt1+Ma	69.62±2.95ab	25.09±2.87ef	51.53	35.11	Synergistic
	Bt1+Ch	62.93±3.80b	31.70±3.80e	57.73	9.00	Additive
	Bt2+Ma	43.79±3.79c	50.84±3.79d	56.64	−22.67	Antagonistic
	Ch	35.51±2.17cd	59.12±2.17cd			
	Ma	29.31±2.19de	65.32±2.19bc			
	Bt2	27.33±0.66de	67.30±0.66c			
	Bt1	22.22±1.11e	72.41±1.11b			
	Control	3.17±0.83f	96.40±1.28a			

of Limonius was induced (Kabaluk *et al.* 2001) by the use of *M. anisopliae* under laboratory conditions. The mortality of larval instars showed a declining trend in all the populations from first to fifth instar supported by Inglis *et al.* (2001) that different developmental stages of insects vary in their susceptibility to infection by entomopathogenic fungi. This could be due to the increase of melanin contents in the cuticle and mid gut of the insects which prevents the penetration of the fungal germ tube (Wilson *et al.* 2001). According to Hafez *et al.* (1997), early larval instars of the potato tuber moth *Phthorimaea operculella* (Z.) (Lepidoptera: Gelechiidae) were more susceptible to *B. bassiana* than older larval stages. On contrary, Vandenberg *et al.* (1998) found that 3rd and 4th instars of the diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae) were more susceptible to entomopathogenic fungi than 2nd instars.

In the current study, efficacy of *B. thuringiensis* toxin decreased with the growth of *H. armigera* larvae; this is confirmed by Herbert & Harper (1985), who noted a decline in the insecticidal activity of *Bt* against *Helicoverpa zea* Boddie (Lepidoptera: Noctudiae) with the growth development of larvae. Similarly, Zehnder & Gelernter (1989) recorded 40–98% mortality of 2nd instars compared with 52% mortality of 3rd instars of the Colorado potato beetle after 96 h with the application of *B. thuringiensis* var. *san diego* (M-ONE). In another study, *B. thuringiensis* at high and low labeled concentrations (1.17 and 7.0 l ha^{-1}) provided fair to excellent control against Colorado potato beetle (Lacey *et al.* 1999). Likewise, Zehnder *et al.* (1992) and Ghidiu & Zehnder (1993) suggested that the appropriate time for the application of *B. thuringiensis* should coincide with the hatching of eggs of Colorado potato beetle and also in the presence of early larval instars.

Chlorantraniliprole gave fair control of all larval instars of *H. armigera* in all the tested populations, but the second instar larvae showed the higher susceptibility in the present study. Cordova *et al.* (2006), Lahm *et al.* (2007) and Temple *et al.* (2009) reported the efficacy of chlorantraniliprole against lepidopteran insect pests at very low concentrations which was further confirmed by Wakil *et al.* (2012) by assessments against *H. armigera,* with promising mortality. The chlorantraniliprole showed a high level of mortality against Cry1Ac susceptible and resistant strains of *H. armigera* (Cao *et al.* 2010), as it increases the esterase and glutathione-S-transferase activities in both strains. Moreover, chlorantraniliprole has the unique mode of action which attacks on the ryanodine receptors in muscle cells resulting in unregulated release of Ca^{+2} and the death of insects (Temple *et al.* 2009).

The results indicate clearly that the mortality was higher when *B. thuringiensis* was combined with *M. anisopliae* and chlorantraniliprole. These findings are in accordance with Lacey *et al.* (1999), who reported the lowest number of adults of *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) in the plots treated with the combination of entomopathogenic

Table 4 Mean mortality and pupation (% ± SE) of fifth instar *H. armigera* larvae from six field populations treated with *B. thuringiensis* (Bt1, Bt2: 0.5 and 1 μg g^{-1}), *M. anisopliae* (Ma: 1.3×10^{6} conidia ml^{-1}) and Chlorantraniliprole (Ch: 0.01 ppm) individually and in combination (Means sharing a common letter within each population do not differ significantly; HSD at 5% significance)

Populations	Treatments	Actual mortality	Pupation	Expected mortality	Co-toxicity factor	Type of interaction
Gujranwala	Bt2+Ch	35.57±2.58a	59.16±2.58e	27.75	28.19	Synergistic
	Bt1+Ma	28.83±1.71ab	64.81±2.56de	22.41	28.62	Synergistic
	Bt1+Ch	27.77±1.11ab	66.96±1.11de	23.49	18.25	Additive
	Bt2+Ma	21.06±2.95bc	73.67±2.95cd	26.68	−21.06	Antagonistic
	Ch	15.67±2.30cd	78.34±2.16c			
	Ma	14.60±1.04cd	80.13±1.04bc			
	Bt2	12.08±2.83cde	82.65±0.89bc			
	Bt1	7.82±2.18de	88.76±1.42b			
	Control	2.65±0.35e	98.67±0.70a			
Sheikhupura	Bt2+Ch	39.56±0.44a	55.20±0.44f	31.71	24.77	Synergistic
	Bt1+Ma	31.41±1.02b	62.77±1.58ef	24.67	27.3	Synergistic
	Bt1+Ch	30.34±0.34b	64.42±0.34e	26.11	16.2	Additive
	Bt2+Ma	22.41±2.75c	72.35±2.75d	30.27	−25.95	Antagonistic
	Ch	17.15±1.80cd	77.61±1.80cd			
	Ma	15.71±0.95cde	79.05±0.95bcd			
	Bt2	14.56±2.10de	80.20±2.10bc			
	Bt1	8.97±1.03ef	86.38±1.62b			
	Control	3.41±0.57f	96.01±0.67a			
Faisalabad	Bt2+Ch	42.24±1.89a	52.62±1.89f	34.12	23.8	Synergistic
	Bt1+Ma	34.81±0.98a	60.05±0.98ef	27.72	25.6	Synergistic
	Bt1+Ch	33.94±2.52ab	63.86±2.65de	28.56	18.82	Additive
	Bt2+Ma	25.65±1.01bc	69.21±1.01cd	33.27	−22.9	Antagonistic
	Ch	18.45±0.96cd	76.41±0.96bc			
	Ma	17.60±2.51cd	77.26±2.51bc			
	Bt2	15.67±2.83d	79.19±2.83b			
	Bt1	10.11±0.11de	84.75±0.11b			
	Control	4.18±0.27e	94.16±0.94a			
Lahore	Bt2+Ch	44.83±2.68a	50.11±2.75f			
	Bt1+Ma	37.29±1.97a	57.57±1.97ef	37.05	21.00	Synergistic
	Bt1+Ch	36.73±1.86a	60.82±1.70de	29.85	24.92	Synergistic
	Bt2+Ma	27.18±2.77b	67.68±2.77cd	31.46	16.77	Additive
	Ch	20.23±0.22bc	75.74±0.22bc	35.44	−23.31	Antagonistic
	Ma	18.62±1.86bc	76.24±1.86bc			
	Bt2	16.82±1.79c	78.04±1.79b			
	Bt1	11.23±1.05cd	83.63±1.05b			
	Control	3.66±0.89f	95.77±0.42a			
Sargodha	Bt2+Ch	47.14±1.77a	47.69±1.77e	38.36	22.87	Synergistic
	Bt1+Ma	40.40±1.59a	52.19±2.53e	33.03	22.31	Synergistic
	Bt1+Ch	39.89±3.30ab	54.94±3.30de	33.61	18.70	Additive
	Bt2+Ma	29.07±1.20bc	65.76±1.20cd	37.78	−23.07	Antagonistic
	Ch	20.39±2.85cd	72.23±2.07bc			
	Ma	19.81±3.42cd	75.02±3.42bc			

Table 4 (continued)

Populations	Treatments	Actual mortality	Pupation	Expected mortality	Co-toxicity factor	Type of interaction
	Bt2	17.97±1.02d	76.86±1.02b			
	Bt1	13.21±1.82de	81.62±1.82b			
	Control	3.80±0.85e	95.34±0.89a			
Rawalpindi	Bt2+Ch	52.38±2.14a	42.45±2.14e	41.41	26.50	Synergistic
	Bt1+Ma	45.55±1.10a	49.28±1.10e	35.60	27.95	Synergistic
	Bt1+Ch	43.69±2.24a	51.14±2.24e	37.34	17.01	Additive
	Bt2+Ma	31.15±1.15b	63.68±1.15d	39.66	−21.46	Antagonistic
	Ch	22.29±2.28bc	69.79±2.68cd			
	Ma	20.54±4.04c	72.83±1.95bc			
	Bt2	19.12±1.24c	74.70±2.03bc			
	Bt1	15.06±1.30c	79.77±1.30b			
	Control	3.32±1.18d	97.36±0.36a			

fungi and *B. thuringiensis*, while the highest number was recorded in control plots. Similarly, Wraight & Ramos (2005) noted the significant reduction in the larval population of Colorado potato beetle in combined treatments of *B. bassiana* and *B. thuringiensis* compared with their individual applications. The mortality of *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae) was increased when *B. bassiana* and *B. thuringiensis* were applied in combination (Lewis *et al.* 1996). The additive interaction is also noted in the present study similar to these findings; in laboratory bioassays Meissle *et al.* (2009) found additive interaction in *Bt* maize and *M. anisopliae* against *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) and this might be due to sublethal damage induced by *B. thuringiensis* toxin that enhanced (Lawo *et al.* 2008) the effectiveness of *M. anisopliae*. Furthermore, Gao *et al.* (2012) also confirmed *Bt–B. bassiana* synergism as interruption of larval feeding by *Bt* intoxication may lead to starvation stress and cause detrimental effects on host physiology and immune response. The possible reasons for synergistic interaction between entomopathogenic fungi and *B. thuringiensis* could be due to starvation, because bacteria may arrest the nutrition of insects (Kryukov *et al.* 2009) and the fungal spores ultimately kill the weakened larvae. The inter-molt period also increased due to starvation and this was the suspected reason for increased susceptibility of the larvae of Colorado potato beetle (Furlong & Groden 2003); also the increased susceptibility of Asian longhorned beetle to *Metarhizium brunneum* Petch (Hypocreales: Clavicipitaceae) was due to the reduced feeding of the insect (Russell *et al.* 2010). On the other hand, in the present study the antagonistic effect of the high dose rate (1 μg g^{-1}) of *B. thuringiensis* in combination with *M. anisopliae* against all the larval instars of *H. armigera* was observed. Ma *et al.* (2008) reported the antagonistic effect of *B. bassiana* and sublethal concentrations of Cry1Ac of *B. thuringiensis* against Asiatic corn borer applied at the rate of 3.2 or 13 μg g^{-1} and 1.8×10^5 and 10^6 conidia ml^{-1}. The antagonistic interaction in this study could be due to the feeding-deterrent effect of a high dose *B. thuringiensis* toxin, which reduces the consumption rate (Lawo *et al.* 2008) of the larvae; additionally, at a higher dose rate the toxin inhibits the conidial germination (Toledo *et al.* 2011). In another study, Costa *et al.* (2001) reported no synergistic interaction against the fourth instar larvae of Colorado potato beetle (*L. decemlineata*) that survived after the treatment of *B. thuringiensis* and entomopathogenic fungi.

This is the first report in which the effectiveness of *M. anisopliae*, *B. thuringiensis* and chlorantraniliprole were tested against different larval instars of *H. armigera* populations originating from different geographical locations in Punjab province (Pakistan). In the light of our findings, the population from Gujranwala appeared to be more resistant to *M. anisopliae*, *B. thuringiensis* and chlorantraniliprole compared with the remaining populations collected from other localities. The variable response exhibited by the field

populations of *H. armigera* to different treatments in the current study could be attributed to the genetic variation and the indiscriminate and repeated excessive spray schedule of insecticides on the crops grown in these particular localities.

Acknowledgments This study is financially supported by projects from the Higher Education Commission (HEC), Islamabad, Pakistan.

References

Ahmad, M., Arif, M. I., & Zahoor, A. (2001). Resistance to carbamate insecticides in *Helicoverpa armigera* (Lepidoptera: Noctudiae) in Pakistan. *Crop Protection, 20*, 427–432.

Ahmad, M., Arif, M. I., & Zahoor, A. (2003). Susceptibility of *Helicoverpa armigera* (Lepidoptera: Noctuidae) to new chemistries in Pakistan. *Crop Protection, 22*, 539–544.

Bravo, A., Gomez, I., Conde, J., Munoz-Garay, C., Sanchez, J., Miranda, R., *et al.* (2004). Oligomerization triggers binding of *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to Cry1Ac δ-endotoxin of *Bacillus thuringiensis* in the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Journal of Economic Entomology, 96*, 1290–1299.

Cao, G., Lu, Q., Zhang, L., Guo, F., Liang, G., Wu, K., *et al.* (2010). Toxicity of chlorantraniliprole to Cry1Ac-susceptible and resistant strains of *Helicoverpa armigera*. *Pesticide Biochemistry and Physiology, 98*, 99–103.

Carner, G. R., & Yearian, W. C. (1989). Development and use of microbial agents for control of *Heliothis* spp. in the USA. In E. G. King, & R. D. Jackson (Eds.), *Proceedings of the workshop on biological control of Heliothis: increasing the effectiveness of natural enemies* (1985, New Delhi, India) (pp. 467–481). Far Eastern Regional Research Office, US Department of Agriculture.

Cherry, A. J., Rabindra, R. J., Parnell, M. A., Geetha, N., Kennedy, J. S., & Grzywacz, D. (2000). Field evaluation of *Helicoverpa armigera* nucleopolyhedrovirus formulations for control of the chickpea pod-borer, *H. armigera* (Hubn.), on chickpea (*Cicer arietinum* var. Shoba) in southern India. *Crop Protection, 19*, 51–60.

Cordova, D., Benner, E. A., Sacher, M. D., Rauh, J. J., Sopa, J. S., Lahm, G. P., *et al.* (2006). Anthranilic diamides: a new class of insecticides with a novel mode of action, ryanodine receptor activation. *Pesticide Biochemistry and Physiology, 84*, 196–214.

Cordova, D., Benner, E. A., Sacher, M. D., Rauh, J. J., Sopa, J. S., Lahm, G. P., *et al.* (2007). Elucidation of the mode of action of Rynaxypyr®, a selective ryanodine receptor activator. In H. Ohkawa, H. Miyagawa, & P. W. Lee (Eds.), *Pesticide chemistry, crop protection, public health, and environmental safety* (pp. 121–126). Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA.

Costa, S. D., Barbercheck, M. E., & Kennedy, G. G. (2001). Mortality of Colorado potato beetle (*Leptinotarsa decemlineata*) after sublethal stress with the CRYIIIA delta-endotoxin of *Bacillus thuringiensis* and subsequent exposure to *Beauveria bassiana*. *Journal of Invertebrate Pathology, 77*, 173–179.

Crecchio, C., & Stotzky, G. (2001). Biodegradation and insecticidal activity of the toxin from *Bacillus thuringiensis* subsp. *kurstaki* bound on complexes of montmorillonite-humic acids-Al hydroxypolymers. *Soil Biology and Biochemistry, 33*, 573–581.

Entwistle, P. F., Cory, J. S., Bailey, M., & Higgs, S. (Eds.). (1993). *Bacillus thuringiensis, an environmental biopesticide: theory and practice*. New York, NY: Wiley.

Furlong, M. J., & Groden, E. (2003). Starvation induced stress and the susceptibility of the Colorado potato beetle, *Leptinotarsa decemlineata*, to infection by *Beauveria bassiana*. *Journal of Invertebrate Pathology, 83*, 127–138.

Gao, Y., Oppert, B., Lord, J. C., Liu, C., & Lei, Z. (2012). *Bacillus thuringiensis* Cry3Aa toxin increases the susceptibility of *Crioceris quatuordecimpunctata* to *Beauveria bassiana* infection. *Journal of Invertebrate Pathology, 109*, 260–263.

Ghidiu, G. M., & Zehnder, G. W. (1993). Timing of the initial spray application of *Bacillus thuringiensis* for control of the Colorado potato beetle (Coleoptera: Chrysomelidae) in potatoes. *Biological Control, 3*, 348–352.

Gunning, R. V., Moores, G. D., & Devonshire, A. L. (1998). Insensitive acetycholinesterase causes resistance to organophosphates in Australian *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). *Pesticide Science, 54*, 319–320.

Hafez, M., Zaki, F. N., Moursy, A., & Sabbour, M. (1997). Biological effects of the entomopathogenic fungus, *Beauveria bassiana* on the potato tuber moth *Phthorimaea operculella* (Zeller). *Journal of Pesticide Science, 70*, 158–159.

Herbert, D. A., & Harper, J. D. (1985). Bioassay of δ-exotoxin of *Bacillus thuringiensis* against *Heliothis zea* larvae. *Journal of Invertebrate Pathology, 46*, 247–250.

Hernández, C. S., Andrew, R., Bel, Y., & Ferre, J. (2005). Isolation and toxicity of *Bacillus thuringiensis* from potato-growing areas in Bolivia. *Journal of Invertebrate Pathology, 88*, 8–16.

Inglis, G. D., Goettel, M. S., Butt, T. M., & Strasser, H. (2001). Use of hyphomycetous fungi for managing insect pests. In T. M. Butt, C. Jackson, & N. Magan (Eds.), *Fungi as biocontrol agents. Progress, problems and potential* (pp. 23–69). Wallingford, UK: CABI Publishing.

Kabaluk, T., Goettel, M., Vernon, B., & Noronha, C. (2001). Evaluation of *Metarhizium anisopliae* as a biological control for wireworms. Organic Agriculture Center of Canada.http://www.organicagcentre.ca/ResearchDatabase/res_biol_ctrl_wireworms.asp; accessed on 20.12.2010.

Kryukov, V. Y., Khodyrev, V. P., Yaroslavtseva, O. N., Kamenova, A. S., Duisembekov, B. A., & Glupov, V. V. (2009). Synergistic action of entomopathogenic hyphomycetes and the bacteria *Bacillus thuringiensis* ssp. *morrisoni*

in the infection of Colorado potato beetle *Leptinotarsa decemlineata*. *Prikladnaya Biokhimiya i Mikrobiologiya, 45*, 571–576.

Lacey, L. A., Horton, D. R., Chauvin, R. L., & Stocker, J. M. (1999). Comparative efficacy of *Beauveria bassiana*, *Bacillus thuringiensis*, and aldicarb for control of Colorado potato beetle in an irrigated desert agroecosystem and their effects on biodiversity. *Entomologia Experimentalis et Applicata, 93*, 189–200.

Lahm, G. P., Stevenson, T. M., Selby, T. P., Freudenberger, J. H., Cordova, D., Flexner, L., *et al.* (2007). Rynaxypyr™: a new insecticidal anthranilic diamide that acts as a potent and selective ryanodine receptor activator. *Bioorganic & Medicinal Chemistry Letters, 17*, 6274–6279.

Lawo, N. C., Mahon, R. J., Milner, R. J., Sarmah, B. K., Higgins, T. J. V., & Romeis, J. (2008). Effectiveness of *Bacillus thuringiensis*-transgenic chickpeas and the entomopathogenic fungus *Metarhizium anisopliae* in controlling *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Applied and Environmental Microbiology, 74*, 4381–4389.

Lewis, L. C., Berry, E. C., Obrycki, J. J., & Bing, L. A. (1996). Aptness of insecticides (*Bacillus thuringiensis* and carbofuran) with endophytic *Beauveria bassiana* in suppressing larval populations of European corn borer. *Agriculture, Ecosystems and Environment, 57*, 27–34.

Ma, X., Liu, X., Ning, X., Zhang, B., Han, F., Guan, X., *et al.* (2008). Effects of *Bacillus thuringiensis* toxin Cry1Ac and *Beauveria bassiana* on Asiatic corn borer (Lepidoptera: Crambidae). *Journal of Invertebrate Pathology, 99*, 123–128.

Mansour, N. A., Eldefrawi, M. E., Toppozada, A., & Zeid, M. (1966). Toxicological studies on the Egyptian Cotton Leafworm, *Prodenia litura* VI potentiation and antagonism of carbamate insecticide. *Journal of Economic Entomology, 59*, 307–311.

Marannino, P., Santiago-Álvarez, C., de Lillo, E., & Quesada-Moraga, E. (2006). A new bioassay method reveals pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana* against early stages of *Capnodis tenebrionis* (Coleoptera; Buprestidae). *Journal of Invertebrate Pathology, 93*, 210–213.

Martin, T., Ochou, G. O., Hala, N. F., Wassal, J., & Waissayre, M. (2000). Pyrethroid resistance in cotton bollworm *Helicoverpa armigera* in West Africa. *Pest Management Science, 56*, 549–554.

Marzban, R., He, Q., Liu, X., & Zhang, Q. (2009). Effects of *Bacillus thuringiensis* toxin Cry1Ac and cytoplasmic polyhedrosis virus of *Helicoverpa armigera* (Hübner) (HaCPV) on cotton bollworm (Lepidoptera: Noctuidae). *Journal of Invertebrate Pathology, 101*, 71–76.

Meissle, M., Pilz, C., & Romeis, J. (2009). Susceptibility of *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) to the entomopathogenic fungus *Metarhizium anisopliae* when feeding on *Bacillus thuringiensis* Cry3Bb1-expressing maize. *Applied and Environmental Microbiology, 75*, 3937–3943.

Nguyen, T. H. N., Borgemeister, C., Poehling, H., & Zimmermann, G. (2007). Laboratory investigations on the potential of entomopathogenic fungi for biocontrol of *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae and pupae. *Biocontrol Science and Technology, 17*, 853–864.

Purwar, J. P., & Sachan, G. C. (2006). Synergistic effect of entomogenous fungi on some insecticides against Bihar hairy caterpillar *Spilarctia oblique* (Lepidoptera: Arctiidae). *Microbiological Research, 161*, 38–42.

Qaim, M., Pray, C. E., & Zilberman, D. (2008). Economic and social considerations in the adoption of *Bt* crops. In J. Romeis, A. M. Shelton, & G. G. Kennedy (Eds.), *Integration of insect-resistant genetically modified crops within IPM programs* (pp. 329–356). Dordrecht, the Netherlands: Springer.

Remadevi, O. K., Sasidharan, T. O., Bhattacharya, J., Vossbrinck, C. R., & Rajan, P. D. (2010). Some pathological effects and transmission potential of a microsporidian isolate (Nosema sp.) from the teak defoliator *Hyblaea puera* (Lepidoptera: Hyblaeidae). *International Journal of Tropical Insect Science, 30*, 138–144.

Rodrigues, R. H., Bechara, I. J., Messias, C. L., & Piedrabuena, A. E. (2005). Effectiveness of *Metarhizium anisopliae* against immature stages of *Anastrepha fraterculus* fruitfly (Diptera: Tephritidae). *Brazilian Journal of Microbiology, 36*, 94–99.

Russell, C. W., Ugine, T. A., & Hajek, A. E. (2010). Interactions between imidacloprid and *Metarhizium brunneum* on adult Asian longhorned beetles (*Anoplophora glabripennis* (Motschulsky)) (Coleoptera: Cerambycidae). *Journal of Invertebrate Pathology, 105*, 305–311.

Sokal, R., & Rohlf, F. J. (1995). *Biometry* (3rd ed.). New York, NY: W. H. Freeman and Company.

Temple, J. H., Pommireddy, P. L., Cook, D. R., Marçon, P., & Leonard, B. R. (2009). Susceptibility of selected Lepidopteran pests to rynaxypyr®, a novel insecticide. *The Journal of Cotton Science, 13*, 23–31.

Toledo, A. V., Alippi, A. M., & de Remes Lenicov, A. M. M. (2011). Growth inhibition of *Beauveria bassiana* by bacteria isolated from the cuticular surface of the corn leafhopper, *Dalbulus maidis* and the planthopper, *Delphacodes kuscheli*, two important vectors of maize pathogens. *Journal of Insect Science, 11*, 29. available online: insectscience.org/11.29.

Vandenberg, J. D., Jackson, M. A., & Lacey, L. A. (1998). Relative efficacy of blastospores and aerial conidia of *Paecilomyces fumosoroseus* against the Russian wheat aphid. *Journal of Invertebrate Pathology, 72*, 181–183.

Vega-Aquino, P., Sanchez-Peña, S., & Blanco, C. A. (2010). Activity of oil-formulated conidia of the fungal entomopathogens *Nomuraea rileyi* and *Isaria tenuipes* against lepidopterous larvae. *Journal of Invertebrate Pathology, 103*, 145–149.

Wakil, W., Ashfaq, M., Ghazanfar, M. U., Afzal, M., & Riasat, T. (2009a). Integrated pest management of *Helicoverpa armigera* in chickpea in rainfed areas of Punjab, Pakistan. *Phytoparasitica, 37*, 415–420.

Wakil, W., Ashfaq, M., Kwon, Y. J., & Ghazanfar, M. U. (2009b). Trends in integrated pest management strategies for the control of *Helicoverpa armigera* (Hübner) caterpillars on chickpea (*Cicer arietinum* L.). *Entomological Research, 39*, 84–88.

Wakil, W., Ghazanfar, M. U., Kwon, Y. J., Qayyum, M. A., & Nasir, F. (2010). Distribution of *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) in tomato fields and its

relationship to weather factors. *Entomological Research, 40*, 290–297.

Wakil, W., Ghazanfar, M. U., Nasir, F., Qayyum, M. A., & Tahir, M. (2012). Insecticidal efficacy of *Azadirachta indica*, *Nucleopolyhedrovirus* and chlorantraniliprole singly or combined against field populations of *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae). *Chilean Journal of Agricultural Research, 72*, 53–61.

Wakil, W., Ghazanfar, M. U., Sahi, S. T., Kwon, Y. J., & Qayyum, M. A. (2011). Effect of modified meridic diet on the development and growth of tomato fruitworm *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Entomological Research, 41*, 88–94.

Wilson, K., Cotter, S. C., Reeson, A. F., & Pell, J. K. (2001). Melanism and disease resistance in insects. *Ecology Letters, 4*, 637–649.

Woodring, J. L., & Kaya, H. K. (1988). *Steinernematid and heterorhabditid nematodes: a handbook of biology and techniques*. Fayetteville, AR USA: Arkansas Agricultural Experiment Station.

Wraight, S. P., & Ramos, M. E. (2005). Synergistic interaction between *Beauveria bassiana* and *Bacillus thuringiensis tenebrionis*-based biopesticides applied against field populations of Colorado potato beetle larvae. *Journal of Invertebrate Pathology, 90*, 139–150.

Zehnder, G. W., & Gelernter, W. D. (1989). Activity of the M-One formulation of a new strain of *Bacillus thuringiensis* against the Colorado potato beetle (Coleoptera: Chrysomelidae): relationship between susceptibility and insect life stage. *Journal of Economic Entomology, 82*, 756–761.

Zehnder, G. W., Ghidiu, G. M., & Speese, J. (1992). Use of the occurrence of peak Colorado potato beetle (Coleoptera: Chrysomelidae) egg hatch for timing of *Bacillus thuringiensis* spray applications in potatoes. *Journal of Economic Entomology, 85*, 281–288.

Zimmermann, G. (1986). The *Galleria* bait method for detection of entomopathogenic fungi in soil. *Journal of Applied Entomology, 102*, 213–215.

Zimmermann, G. (2007). Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Science and Technology, 17*, 879–920.