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ORIGINAL ARTICLE

Interaction between the entomopathogenic bacterium *Bacillus thuringiensis* subsp. *kurstaki* and two entomopathogenic fungi in bio-control of *Sesamia nonagrioides* (Lefebvre) (Lepidoptera: Noctuidae)

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Abstract The interactions between the entomopathogenic bacterium *Bacillus thuringiensis* ssp. *kurstaki* and two entomopathogenic fungi *Beauveria bassiana* Balsamo (Vuillemin) (Hypocreales: Cordycipitaceae) and *Metarhizium robertsii* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) were examined on larvae of *Sesamia nonagrioides* (Lefebvre) (Lepidoptera: Noctuidae) in 8, 13 and 16 days post-treatment intervals. An overall positive interaction between the pathogens was observed and the larval mortality at 16 days was 56–100 % exposed to *M. robertsii* combined with *B. thuringiensis* subsp. *kurstaki*, whereas *B. bassiana* combined with *B. thuringiensis* ssp. *kurstaki* killed 54–100 % of exposed larvae. After 8 days, in 6 of the combinations, we found an additive relationship between the pathogens, whereas, a negative interaction was observed in 10 of them. In contrast, after 13 days, in 2 of the combinations the positive interaction could be considered as synergistic between pathogens, in 10 as additive, and in only 4 as negative. Finally, after 16 days, in 11 of the combinations we found an additive connection between the pathogens, wheras a negative interaction was seen in 5. Applying both pathogens simultaneously offers a method of *Sesamia nonagrioides* control that could be more effective than using each pathogen separately.

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Introduction

Within the Mediterranean Basin (below the 45°N parallel) (Eizaguirre and Fantinou 2012), *Sesamia nonagrioides* (Lefébvre) (Lepidoptera: Noctuidae), commonly known as the Mediterranean corn stalk borer, was first described in 1824 in Sicily (Lefebvre 1827) and is considered one of the most important pests of maize and sweet sorghum (Tsitsipis 1990; Dimas et al. 2007). *Sesamia nonagrioides* is multivoltine in Greece, with three to four generations annually. Adult emergence is initiated at the beginning of April (Tsitsipis 1990; Eizaguirre and Fantinou 2012). Adult life lasts 5–10 days, with the males emerging slightly earlier than the females (Lopez et al. 2003). Females lay their eggs in a few to more than 100 clutches under a leaf sheath (Tsitsipis 1990). Larvae emerge within a week, depending on temperature, commence feeding on the leaves, and later bore into the stalk or ear (Tsitsipis 1990). Although one larva may occupy one root, several larvae may be found in a stem (Butron et al. 1999).

Damage is mostly done in the second and subsequent generations by larvae that enter the maize stalk after hatching and feed on the stalk pith (Malvar et al. 2008). Maize yield is affected by corn borer tunneling, which interferes with assimilation movement and increases the risk of stalk lodging. Yield loss due to ear attack is less important than yield reduction associated with stalk tunneling; this type of damage has been described as an important factor of favoring high levels of fumonisins in maize kernels

(Avantaggiato et al. 2002; Butron et al. 2006). *Sesamia nonagrioides* larvae enter the plant preferentially through the ear shank (Velasco et al. 2002).

Currently, the control of *Sesamia nonagrioides* in Greece is primarily based on the use of synthetic insecticides, often with poor effectiveness because of the cryptic feeding behavior of the pest. Thus, testing and evaluation of alternative methods for insect control is imperative. Application of microorganisms and especially fungi is very promising and has provided satisfactory results in the control of harmful insect populations (Hajek et al. 2007; St. Leger et al. 2011).

Several asexual fungal species are associated with arthropods, especially with insects. Specifically, 700 species and more are pathogenic to insects but only a dozen have been exploited for insect control (St. Leger et al. 2011). Among these species, *Beauveria* and *Metarhizium* are important genera being used for insect management. The white muscardine fungus, *Beauveria bassiana* Balsamo (Vuillemin), and the green muscardine fungus, *Metarhizium robertsii* (Metchnikoff) Sorokin, formerly known as *M. anisopliae* var. *anisopliae* (Bischoff et al. 2009), have been recorded to infect 500 (Moore and Prior 1996) or over 700 (Goettel et al. 2000) host species belonging to the orders of Lepidoptera, Hemiptera, Homoptera, Orthoptera and Diptera.

As an alternative to synthetic insecticides, beside the use of entomopathogenic fungi, are formulations based on *Bacillus thuringiensis* (Berliner) which have been used to control insect pests for decades. These formulations are used since they are environmentally friendly, and harmless to humans and other vertebrates (IPSC-WHO 2000). *Bacillus thuringiensis* formulations contain bacterial spores and several different endotoxins, including Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, and Cry2B, active against Lepidoptera including *Sesamia nonagrioides* (Dias et al. 2005; Eizaguirre et al. 2005; Ma et al. 2008; Gonzalez-Cabrera et al. 2011).

This is the first attempt in which combined treatments of entomopathogenic microorganisms are evaluated as control strategies against *Sesamia nonagrioides*. The objective of the present study was to evaluate the interaction between *Beauveria bassiana* and *Metarhizium robertsii* with the entomopathogenic bacterium *Bacillus thuringiensis* ssp. *kurstaki* with respect to *Sesamia nonagrioides* larval mortality in the laboratory. This type of interaction between entomopathogenic fungi and entomopathogenic bacterial infections has not yet been evaluated in terms of pest control efficacy; understanding the three-way system *Sesamia nonagrioides*–entomopathogenic fungus–entomopathogenic bacterium might allow an increase in pest control efficiency and improve management strategies.

Materials and methods

Insect

Sesamia nonagrioides larvae were initially collected from crop fields in Isoma, Achaea (38°10′N, 21°70′E) and were reared on artificial substrate in laboratory conditions (Plant Physiology laboratory, Department of Biology, University of Patras, Greece). The artificial diet contained 1 g potassium sorbate, 2 g nipagin, 3 g ascorbic acid, 4 g vitamin mixture Vanderzant for insects, 25 g soy flour, 50 g brewer yeast, 25 g maize germ, 400 g cellulose, 40 ml HCl (2 N), and 700 ml water. All stages of insect development were performed in a room with constant temperature 25±1 °C, humidity 60–70 %, and a photoperiod 16:8 h light:dark (Tsitsipis 1984).

Bioassays

Fungi

Fungal isolates (*Metarhizium robertsii* Elateridae1, *Beauveria bassiana* IGE3) were derived from the collection of the Benaki Phytopathological Institute. The above strains were selected from among five strains (two *Metarhizium robertsii*, three *Beauveria bassiana*) since they showed a high level of mortality against *Sesamia nonagrioides* larvae in previous experiments. In order to prepare appropriate suspensions for the experiments, the isolates were grown in 9-cm Petri dishes with Sabouraud Dextrose Agar (Sigma–Aldrich) and left in the dark for 15 days at 25 °C±1. The Petri dishes were sealed with Parafilm® to avoid contamination. For each dose, fresh conidia were collected from the cultures after 15 days. Conidia were harvested from the SDA culture by scraping them off the medium surface with a loop needle and transferring them to a 500-ml glass beaker with 100 ml sterile distilled water containing 0,05 % Tergitol® NP9. The conidia suspension was filtered across several layers of sterile cloth and prepared by mixing the solution with a magnetic stirrer for 5 min (Quesada-Moraga et al. 2007). Subsequently, a Neubauer hemocytometer was used to determine the appropriate conidia doses under a phase contrast microscope at ×400 magnification. Conidia germination was estimated at 95 %. This was assessed by examining 100 conidia using a compound microscope at ×40 magnification after they had been incubated for 24 h on SDA at 25 °C in absolute darkness.

Bacteria

For the bacterial treatments, Bactospeine® 32 WG, a microbial insecticide from *Bacillus thuringiensis* ssp. *kurstaki* (Hellafram, Greece), formulated as granules and wet table powder (WG) with 32,000 IU/mg potency was used. Aqueous suspensions of each dose were prepared at the

appropriate concentrations. The powder was mixed with water in a sterilize Erlenmeyer flask (100 ml) using a sterilized spatula. Then, aqueous suspensions were prepared by mixing the solution with a magnetic stirrer for 3 min.

Virulence estimation against *S. nonagrioides*

To determine the virulence of the entomopathogenic fungi (*Metarhizium robertsii* Elateridae1, *Beauveria bassiana* IGE3) against *Sesamia nonagrioides* larvae, 26-day-old larvae were treated with three concentrations of conidia. To establish a dose–mortality relationship, the following concentrations of entomopathogenic fungus were used: 10^3, 10^4, 10^5 conidia/ml. Fifty larvae, 26 days old (10 larvae in 5 replications), were used for each concentration. Direct spray was applied. A batch of 10 *Sesamia nonagrioides* larvae was placed in a sterile 9-cm-diameter Petri dish and were sprayed with a 10-ml conidial suspension using a Potter spray tower (Burkard Manufacturing, England), then directly transferred to plastic containers (1,000 ml) and subsequently placed in the artificial diet. All treated larvae were supplied with the standard artificial diet that contained no anti-fungal preservatives. Treated larvae were maintained at 25±1 °C, humidity 60–70 % and photoperiod 16:8 (L/D). Larvae were observed daily and mortality was recorded for 16 days. Dead larvae were removed from the plastic containers, which were superficially disinfested to avoid fungal saprophytic growth. Subsequently, the sterilized larvae were kept individually in Petri dishes with a moist filter paper until mycelia appeared. Mycosis or cadavers showing external mycelia growth were determined by examining each cadaver using a stereomicroscope.

The effects of *Bacillus thuringiensis* ssp. *kurstaki* on the *Sesamia nonagrioides* larvae were examined by treating with three concentrations of aqueous suspensions (0.5 g/100 ml, 0.25 g/100 ml, and 0.125 g/100 ml), number, the age of individuals, replications, and spray procedures were as described above, in order to adopt similar conditions as for the fungal trials, since *Bacillus thuringiensis* is a "stomach action" microbial agent that has to be sprayed on the plant substrate or on the artificial diet on which the insects are fed and to avoid a watery composition of artificial diet, which could cause the death of larvae by drowning. The toxins (Bactospeine® 32 WG contains Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, Cry2B, and spores), reached the "stomach" of the constantly moving larvae by ingestion of inoculum droplets created from the solution on the surface of the Petri dishes during spraying and secondly by mixing the "wet" larvae with the artificial diet in the containers to facilitate spread of the toxins.

To study the combined effect of entomopathogenic fungi (*Metarhizium robertsii* Elateridae1, *Beauveria bassiana* IGE3) with *Bacillus thuringiensis* ssp. *kurstaki,* the larvae were treated with eight different suspensions of each entomopathogenic fungus and *Bacillus thuringiensis* subsp. *kurstaki* (10^5–0.5 g, 10^5–0.25 g, 10^5–0.125 g, 10^4–0.5 g, 10^4–0.25 g, 10^3–0.5 g, 10^3–0.25 g, 10^3–0.125 g). The number and age of individuals, replications, and spray procedures were as described above. The spray sequence included an initial spray of the larvae with the first pathogen (entomopathogenic fungus or entomopathogenic bacterium) and a subsequent spray with the second pathogen (entomopathogenic fungus or entomopathogenic bacterium). The sequence of pathogens was rotated in every replicate for each combined solution.

Statistical analysis

Corrected percent mortality was calculated using Abbott's formula (Abbott 1925) and prior to analysis these values were arcsine-transformed. Data were then analyzed by means of two-way ANOVA (Univariate) using the general linear model of the PASW (v.18.0.3; SPSS, IL, USA) (SAS Institute 2011). In the case of significant F values, means were compared using the Bonferroni test. The significance level was set at $P<0.05$. The survival times of *Sesamia nonagrioides* larvae in combined suspensions of pathogens were calculated using Kaplan–Meier survival analysis and the Gehan–Breslow test, and pairwise comparisons were made using the Gehan–Breslow test.

Mathematical estimation

Interaction between pathogens was estimated using the formula of Robertson and Preisler: $P_E = P_0 + (1 - P_0) * (P_1) + (1 - P_0) * (1 - P_1) * (P_2)$, where P_E is the expected mortality induced by the combination of the two pathogens, P_0 the mortality of the control, P_1 the mortality caused by the first pathogen, and P_2 the mortality caused by the second pathogen. Distribution was determined by the chi-square formula: $x^2 = (L_0 - L_E)^2/L_E + (D_0 - D_E)^2/D_E$ where L_0 is the number of live larvae, D_0 the number of dead larvae, L_E the expected number of live larvae, and D_E the expected number of dead larvae. The formula was used to test the hypothesis independent–simultaneous relationship (1df, $P=0.05$). If $\chi^2<3.84$, the ratio is defined as additive if $\chi^2>3.84$, and the mortality observed higher than expected, the relationship is defined as synergistic, while if $\chi^2>3.84$ and the mortality observed less than expected, the relationship is defined as competitive (Rahman et al. 2010). The above formula was used to estimate the interaction of pathogens after 8, 13, and 16 days.

Results

All pathogens tested against *Sesamia nonagrioides* larvae induced various levels of mortality. Mortality caused by *Bacillus thuringiensis* ssp. *kurstaki* resulted in both time-

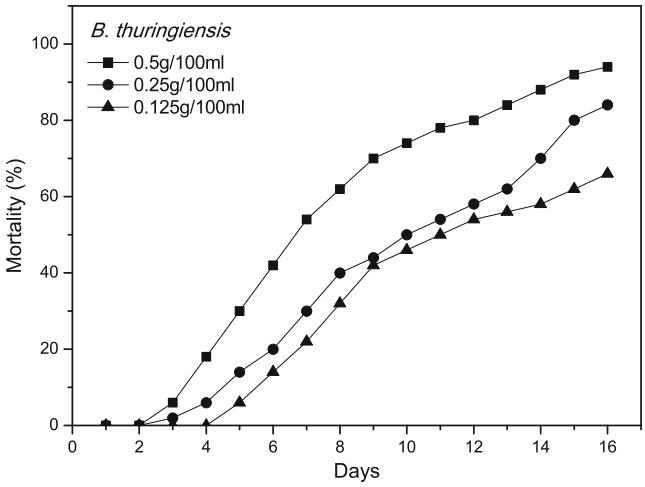

Fig 1 Mortality (%) of *S. nonagrioides* larvae treated with aqueous solution of *B. thuringiensis* ssp. *kurstaki* (n=50/dose, 25 °C)

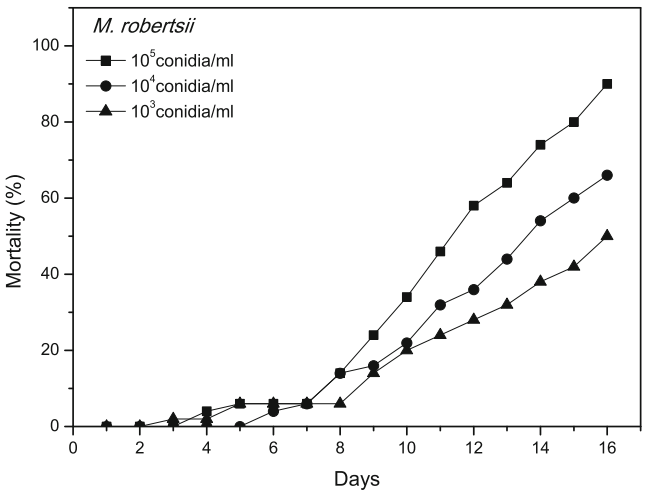

Fig 3 Mortality (%) of *S. nonagrioides* larvae treated with conidial suspensions of *B. bassiana* (n=50/dose, 25 °C)

and dose-dependent mortality (Fig. 1), given that it caused 66, 84 and 94 % larval mortality, at 0.125, 0.25 and 0.5 g/100 ml (F=2.006, df=2, P=0.152), respectively. Larval mortality caused after 16 days exposure to *Beauveria bassiana* was measured 72, 86 and 96 % at doses of 10^3, 10^4, and 10^5 conidia/ml (F=0.481, df=2, P=0.621), respectively (Fig. 2), whereas respective values for *Metarhizium robertsii* were 50 %, 66 % and 90 % (F=1.699, df=2, P=0.194) (Fig. 3). Mortality of control larvae was 28 % after 16 days. Significant differences in mortality were detected between the treatments (F=11.174, df=3, P<0.001).

The combined suspensions of pathogens used against larvae of *Sesamia nonagrioides* during the present study were *Bacillus thuringiensis* ssp. *kurstaki* with *Beauveria bassiana* and *Bacillus thuringiensis* ssp. *kurstaki* with *Metarhizium robertsii* in various doses and combinations (overall 16 treatments). The larval mortality was 56–100 % exposed to

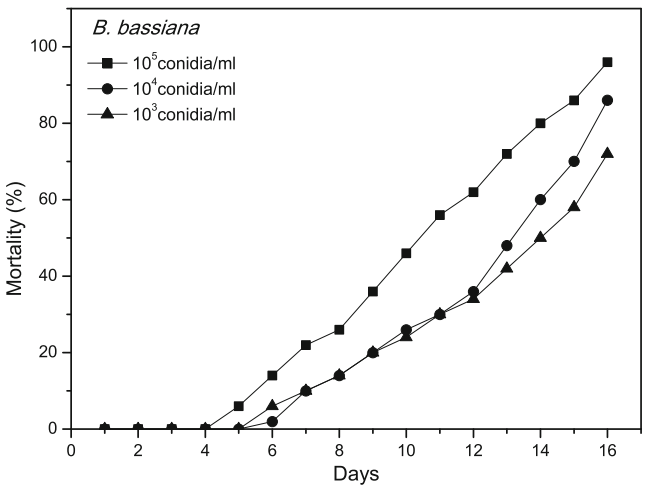

Fig 2 Mortality (%) of *S. nonagrioides* larvae treated wit conidial suspensions of *M. robertsii* (n=50/dose, 25 °C)

Metarhizium robertsii combined with *Bacillus thuringiensis* ssp. *kurstaki* (Fig. 4d–f), whereas *Beauveria bassiana* combined with *Bacillus thuringiensis* ssp. *kurstaki* killed 54–100 % of exposed larvae (Fig. 4a–c). All the bioassays were performed concurrently in order to achieve identical conditions for all the treatments. Unfortunately, the larvae population was not big enough to provide the statistically appropriate number of individuals for all the combinations. We decided to keep statistical confidence and exclude an intermediate dose, as this would be the least significant for possible trends and patterns evaluated from the experiment.

Significant differences in mortality were detected between the treatments (F=5.247, df=2, P=0.006) and between doses (F=4.701, df=7, P<0.001). Kaplan–Meier survival analysis (Gehan–Breslow test) showed that the median overall survival time for the larvae was 9±0.3 days for *Bacillus thuringiensis* ssp. *kurstaki* with *Beauveria bassiana* and 10±0.2 days for *Metarhizium robertsii* combined with *Bacillus thuringiensis* ssp. *kurstaki*. Significant differences in mortality were detected between combined doses in Kaplan–Meier survival analysis (Gehan–Breslow test and Pairwise Comparisons Breslow test) (F=256.576, df=7, P<0.001). Particularly, the Gehan–Breslow test displayed significant differences in mortality between the doses, whereas no significant differences were found in the combinations where the concentration of *Bacillus thuringiensis* ssp. *kurstaki* was high (Table 1).

The aforementioned specific time points were selected to estimate the interactions between treatments, based on the Kaplan–Meier analysis result. Therefore, three specific points were selected, of which the first was the median length time of our experiment, slightly lower than the median survival time of *Sesamia nonagrioides* larvae, and the next two were longer than the median survival time of larvae (Kaplan–Meier analysis). The first of the two larger time points is equivalent to the

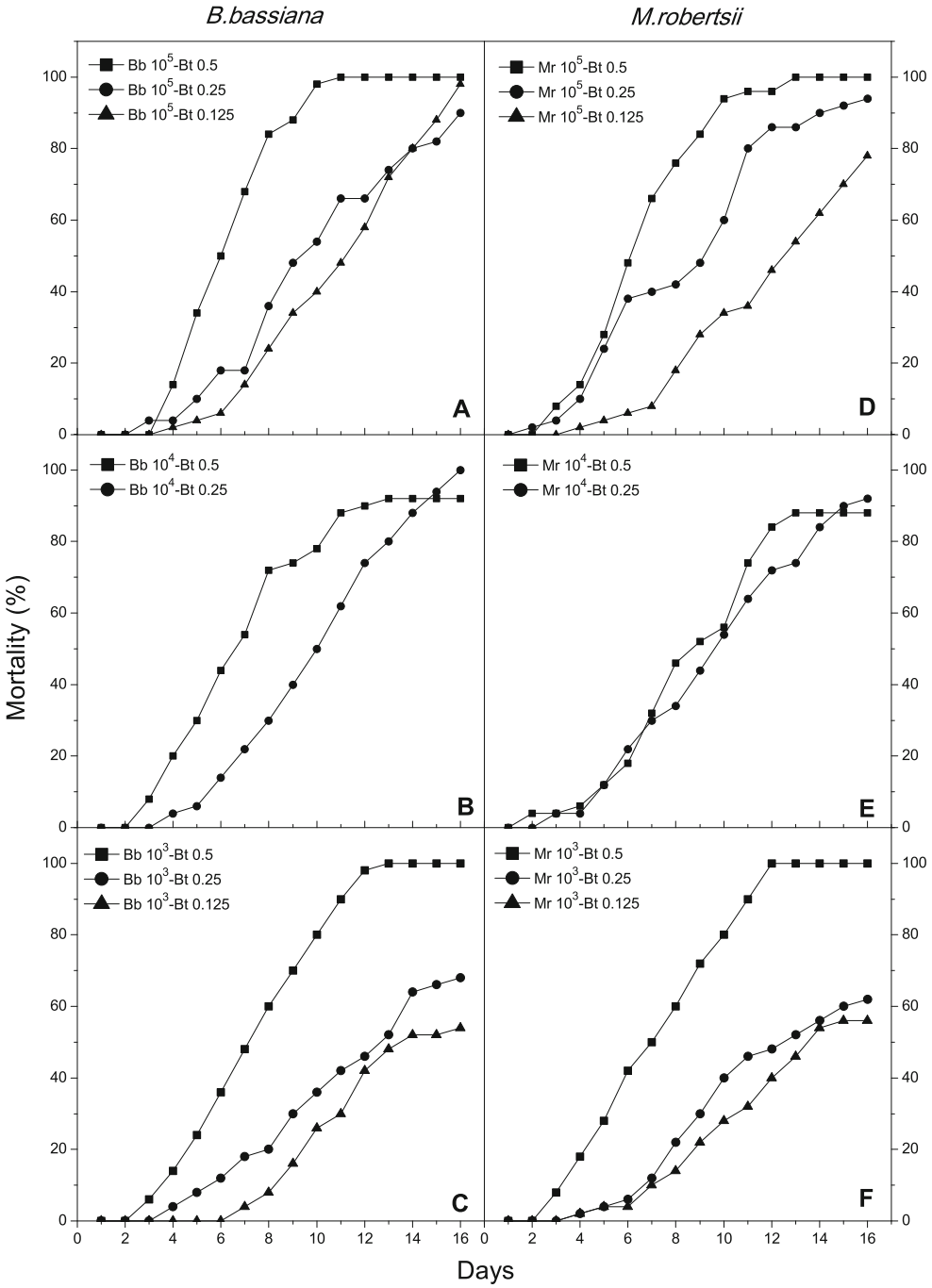

Fig 4 Mortality (%) of *S. nonagrioides* larvae treated with combined pathogens in laboratory condition (n=50/ combine dose, 25 °C)

end of our experiment and the other point is the median between the median survival time of larvae and the termination of our measurements. Based on the above considerations, the time points selected were 8, 13, and 16 days.

At 8 days, the interaction of the pathogens was additive in 6 treatments and competitive in 12. At 13 days, there was synergistic action in 2 treatments, additive in 10 and competitive in 4. Finally, at 16 days, in 11 treatments the interaction was additive, whereas there was a negative interaction that could be described as competitive in 5 of them (Table 2).

Discussion

Several studies have described interactions between different pathogens within the same host (Lewis et al. 1996; Bauer et al. 1998; Inglis et al. 2001; Thomas et al. 2003; Wraight and Ramos 2005; Ma et al. 2008). Generally, insect infections by more than one pathogen usually lead to an increase in the numbers of individuals killed, particularly when the infections are separated by a time interval of several days (Jacques and Morris 1981). Lewis et al. (2002) showed that the use of *Beauveria bassiana* in

Table 1 Breslow Pairwise Comparisons (Generalized Wilcoxon) between the combined doses (Kaplan–Meier survival analysis, Gehan–Breslow test, $P=0.05$)

Fungus		Chi square							
		10^5 conidia/ml			10^4 conidia/ml		10^3 conidia/ml		
	Bt	0.5 g/ 100 ml	0.25 g/ 100 ml	0.125 g/ 100 ml	0.5 g/ 100 ml	0.25 g/ 100 ml	0.5 g/ 100 ml	0.25 g/ 100 ml	0.125 g/ 100 ml
10^5 conidia/ml	0.5 g/100 ml		33,224*	96,919*	12,621*	50,621*	5,074	87,842*	126,881*
	0.25 g/100 ml	33,224*		13,565*	3,779	0,610	14,237*	18,796*	40,554*
	0.125 g/100 ml	96,919*	13,565*		37,323*	11,439*	64,035*	1,882	10,963*
10^4 conidia/ml	0.5 g/100 ml	12,621*	3,779*	37,323*		9,330*	2,101	35,105*	62,403*
	0.25 g/100 ml	50,621*	0,610	11,439*	9,330*		25,107*	16,552*	37,613*
10^3 conidia/ml	0.5 g/100 ml	5,074	14,237*	64,035*	2,101	25,107*		60,072*	95,387*
	0.25 g/100 ml	87,842*	18,796*	1,882	35,105*	16,552*	60,072*		3,041
	0.125 g/100 ml	126,881*	40,554*	10,963*	62,403*	37,613*	95,387*	3,041	

Bt Bacillus thuringiensis

*Significant difference at the 0.05 level

combination with *Bacillus thuringiensis* ssp. *kurstaki* reduced the population of *Ostrinia nubilalis*. In our experiments, we reached the same conclusion. The survival test results showed that use of two pathogens simultaneously reduced further the population of *Sesamia nonagrioides* larvae. Also, our results showed that the combination of the two pathogens increased the mortality of *Sesamia nonagrioides* larvae during the course of time.

Pevling and Weyrich (1992) and Mietkiewski and Gorski (1995) reported that when *Beauveria bassiana* and *Metarhizium robertsii* were used concurrently with other biological insecticides, they sometimes exhibited synergy, sometimes competitiveness, and sometimes a neutral interaction. Based on our results after 8 days, the combined treatments exhibited negative interactions, which could be considered as a competitive relationship between the pathogens. These interactions may affect pest mortality, reducing observed host mortality as compared to the single pathogen infections, or interactions may vary with genotype, dose, and order of infection (Bauer et al. 1998; Thomas et al. 2003). This may be a result of differences in the infection route of the two pathogens. Entomopathogenic fungi penetrate primarily through the external cuticle and enter the hemocoel, where they continue to grow and release a number of toxic compounds. On the other hand, *B. thuringiensis* toxin enters the insect via ingestion and binds to glycoprotein receptors of the target insect midgut epithelium, where they disrupt the cytoplasmic membrane, leading to cell lysis (Hilder and Boulter 1999). The route of infection of entomopathogenic fungi and the primary site of activity of d-endotoxin of *Bacillus thuringiensis* are spatially separated within an insect.

Application of *Bacillus thuringiensis* ssp. *kurstaki* in combination with the fungus *Beauveria bassiana* has shown an independent action against *Ostrinia nubilalis* (Lewis and Bing 1991; Lewis et al. 2002), *Leptinotarsa decemlineata* (Costa et al. 2001), and *Ephestia kuehniella* (Sandner and Cichy 1967) in former studies. In contrast, Ma et al. (2008) reported on the possibility of synergy between the two pathogens when they are applied in combination against *Ostrinia furnacalis* (Guenée). Also, Wraight and Ramos (2005) observed a synergistic interaction when *Beauveria bassiana* and *Bacillus thuringiensis* ssp. *tenebrionis* were applied combined (tank-mixed) against the *Leptinotarsa decemlineata* in small potato plots.

In our experiments, after 13 and 16 days, the combined treatments exhibited a positive interaction, mostly additive, as synergistic in some combinations. Synergism of two pathogens that are administered with the same exposure method might be observed when in combined treatments one pathogen increases, directly or indirectly, the insecticidal activity of the other. The synergistic or additive effect in insect infection with bacterial–fungal pathogens is most likely determined when three main conditions are met. Firstly, bacterial infection directly kills insects within a few days and the remaining individuals are then killed by mycosis, giving an effect of accelerated mortality. Secondly, the intestinal dysfunction and general intoxication caused by bacteria interfere with insect feeding, delay their growth, lengthen the intermolt period, and impair metamorphosis during molts (sometimes larvae are unable to shed the old chitin cover). Delayed growth and molting assist fungal hyphae entrance in the cuticle and haemolymph and

Table 2 Percentage mean mortality of *S. nonagrioides* larvae at 8, 13, and 16 days treated with *B. bassiana* and *M. robertsii* combine with *B. thuringiensis* ssp. *kurstaki* (Bt) in several combinations (*A* additive, *C* competitive, *S* synergistic) (n=50)

Dose		Mortality (%)		X^2	Interaction	Mortality (%)		X^2	Interaction	Mortality (%)		X^2	Interaction
Fungus (conidia/ml)	Bt (g/100 ml)	Observed	Expected[a]	(1 *df*, P=0.05)		Observed	Expected[a]	(1 *df*; P=0.05)		Observed	Expected[a]	(1 *df*; P=0.05)	
		8 days				13 days				16 days			
M. robertsii													
10^5	0.5	76	73	1,831	A	100	96	2,631	A	100	99	0,505	A
	0.25	42	48	0,721	A	86	85	0,148	A	94	99	0,521	A
	0.125	18	46	15,781	C	54	92	54,734	C	78	97	82,687	C
10^4	0.5	46	70	13,714	C	88	93	1,086	A	88	95	3,209	A
	0.25	34	52	6,49	C	74	84	3,720	A	92	96	2,088	A
10^3	0.5	60	67	2,38	A	100	92	5,555	S	100	98	1,02	A
	0.25	22	48	13,541	C	52	83	2,711	A	62	94	90,78	C
	0.125	14	32	19,333	C	46	77	29,064	C	56	88	48,484	C
B. bassiana													
10^5	0.5	84	67	3,571	A	100	97	2,083	A	100	99	0,1	A
	0.25	36	57	8,116	C	74	78	0,116	A	90	97	1,418	A
	0.125	24	44	8,271	C	72	93	18	C	98	98	0,000	A
10^4	0.5	72	70	0,095	A	92	94	0,354	A	92	97	2,088	A
	0.25	30	52	9,695	C	80	85	0,6	A	100	98	0,813	A
10^3	0.5	60	70	0,802	A	100	93	4,347	S	100	98	0,525	A
	0.25	20	52	20,512	C	52	83	1,086	A	68	96	102,083	C
	0.125	8	38	8,092	C	48	80	32	C	54	92	98,097	C

[a] Expected mortality calculated according to Robertson and Preisler (Abdel Rahman et al. 2010)

enhance mycosis development. In this case, the effect of accelerated mortality is similar to starvation of insects. For example, Furlong and Groden (2001) have demonstrated that a 24-h starvation of Colorado potato beetle larvae increases its sensitivity to *Beauveria bassiana*. Thirdly, it is possible that fungal infection elevates larval susceptibility to bacterial infection (Kryukov et al. 2009).

Finally, from our observations and results, it becomes obvious that *Bacillus thuringiensis* ssp. *kurstaki* toxins are responsible for larval mortality, and the fungus, at all concentrations, subsequently kills all remaining "sickened" larvae. This occurs at the high (0.5 g/100 ml) dose of *B. thuringiensis* ssp. *kurstaki* without the dead larvae showing mycosis signs, except in some cases at the high (10^5 conidia/ml) concentration of the fungus. Therefore, the interaction was found additive. On the other hand, the mid- (0.25 g) to low (0.125 g) doses of *B. thuringiensis* ssp. *kurstaki* were responsible for weakening the larval "vital functions", making them more sensitive to fungus penetration, at all concentrations, and in the end the fungus infection was responsible for larval mortality. In this case, at the mid- to –low doses of *Bacillus thuringiensis* ssp. *kurstaki* and high to mid- (10^4 conidia/ml) concentrations of fungus, the dead larvae showed toxemia and external mycelia growth (in some cases concurrently). This led mostly to an additive interaction expected in the combined dose, *Metarhizium robertsii* 10^5– *B. thuringiensis* ssp. *kurstaki* 0.125 g, where the interaction that occurred was competitive. Lastly, the mid- to low doses of *Bacillus thuringiensis* ssp. *kurstaki* affected the larvae as mentioned above, but the rather low concentration (10^3 conidia/ml) of the fungus did not contribute enough to larvae mortality, so the interaction was in the end competitive. In this situation, in some cases larvae exhibited toxemia but not mycelial growth.

Under natural conditions, insect infections involving more than one pathogen are common. Therefore, there is a prominent need to understand how pathogens interact with each other. In mixed infections, it is possible that the efficacy of one or both pathogens may be improved, suppressed, or enhanced. Our results demonstrated that the combination of *Bacillus thuringiensis* ssp. *kurstaki* with an entomopathogenic fungus may increase *Sesamia nonagrioides* larval mortality. An overall positive interaction between the two pathogens was noticed in terms of larval mortality. Applying both entomopathogenic microorganisms offers a method of *Sesamia nonagrioides* control that could be more effective than using each pathogen alone, providing that increased mortality in the present study can be reproduced under field conditions. Nevertheless, further studies on the mechanism of toxicity of these combinations in lepidopterans are needed.

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