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The effect of *Metarhizium robertsii* and *Bacillus thuringiensis* against *Helicoverpa armigera* (*Hübner*) (*Lepidoptera: Noctuidae*)

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Abstract: Susceptibility of *Helicoverpa armigera* populations to *Metarhizium robertsii* and *Bacillus thuringiensis* (Bt) were evaluated under laboratory conditions. *Helicoverpa armigera* larvae were treated individually and in combination with a single dose rate of *B. thuringiensis* (Bt: 1 ml/L) and three conidial suspensions viz. $1x10^3$, $1x10^5$ and $1x10^7$ spores/ml of *M. robertsii*. Compared with the untreated checks, pupation, adult emergence and egg eclosion of both instars larvae were significantly reduced in combined application treatment of *M. robertsii* ($1x10^7$ spores/ml) and *B. thuringiensis* (1 ml/L). The results indicate that the entomopathogenic fungi and the insecticidal protein produced by *B. thuringiensis* can be used as potential biocontrol agents for the management of maize stem borer.

Key words: *Bacillus thuringiensis*, biological control, *Helicoverpa armigera*, *Metarhizium robertsii*, tomato

1. Introduction

Tomato (*Solanum lycopersicum* L., Solanaceae) is one of the most common and nutritious vegetable crops in Greece. Tomato is very susceptible to insect attack throughout its life cycle, from seedling to fruiting stage. This crop is attacked by several insect species in Greece, among which, the highly polyphagous tomato fruit borer, *Helicoverpa* (=*Heliothis*) *armigera* (Hübner) (Lepidoptera: Noctuidae) which is the most significant (Akbulut *et al*. 2003). Its larvae have been reported affecting more than 60 species of cultivated and wild plants belonging to ~ 67 host families (Pogue 2004). Furthermore, this herbivore insect can cause losses to various other economically important crops, such as cotton, sorghum, maize, tomato, and several

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leguminous, ornamental and tree plant species (Moral Garcia 2006). *H. armigera* is geographically widespread, being present in Europe, Asia, Africa and Oceania (Akbulut *et al*. 2003). Its larvae feed on the leaves, stems, green color fruits, causing Post-harvest damage of tomato produce (Wang & Li 1984). The use of microorganisms provide an alternate to chemical insecticides with increased environmental safety and pest selectivity; hence can be used either uniqly or integratedly with other pest control tactics. Moreover, microbial agents also facilitate the survival of beneficial fauna due to their high target specificity. Entomopathogenic fungus *Metarhizium robertsii* and bacterium *Bacillus thuringiensis* (Bt) have an important part in crop protection with effective and ecologically sound solution to pest problems (Schnepf *et al*., 1998; Zimmermann, 2007). Entomopathogenic fungus *M. robertsii* serves as an attractive alternative tool for the management of Lepidopterous insect pests (Carner and Yearian, 1989). These fungi mainly use propagules such as conidia, blastospores or hyphae which infect the host by direct contact however; secondary infection took place by horizontal transmission of spores from mycosed cadavers (Shan and Feng, 2010). Unlike other microbial agents which needs to be ingested to show their action, the mycospores upon contact adhere to and infiltrate the cuticle thus grow internally and produce different toxins that kill the insect pests (Scholte *et al*., 2003; Zimmermann, 2007). *B. thuringiensis* (Bt) is a ubiquitous gram-positive endospore forming soil inhabiting rod-shaped bacterium that produces parasporal crystalline proteinaceous inclusions (Cry and Cyt toxins) during its sporulation and stationary growth phases (Kumar, 2003; Bravo *et al*., 2005). The primary action of Cry toxins in insect body is the cessation of feeding due to paralysis of the mouthparts and gut, leading to the formation of pores in the apical microvilli membrane of the target cells which results in the lysis of midgut epithelial cells and septicemia (Hilder and Boulter, 1999; Bravo *et al*., 2005). The specific and narrow spectrum of action of these toxic crystal proteins against lepidopteran, dipteran and coleopteran insects (Bravo *et al*., 2007; Van Frankenhuyzen, 2009) recommends their use in food and other sensitive crops where chemicals may cause severe hazardous effects (Kumar *et al.*, 1996; Schnepf *et al*., 1998; Bates *et al*., 2005). Studies revealed that in case of lepidopterous pests, commercially available *B. thuringiensis* intoxication enhanced the efficacy of entomopathogenic fungi when used in an integrated fashion (Gao *et al*., 2012; Wakil *et al*., 2013). The enhanced synergistic effect between the two could be due to larval starvation, because bacteria may arrest the nutrition of insects and the fungal spores ultimately kill the weakened worms quickly (Kryukov *et al*., 2009). Keeping in view the significance of these promising alternatives, the current experiment was planned to evaluate the alone and combined effects of *M. robertsii* and *B. thuringiensis* on pupation, adult emergence, and egg eclosion of 2^{nd} and 4^{th} larval instars of *H. armigera*.

2. Materials and Methods

Insect culture

In this study, *H. armigera* individuals, initially collected from tomato fields in Mirtia, Hlia, Greece (37.702267, 21.359392), were reared on artificial substrate in laboratory conditions as reported by Özgur *et al*. 2009. All stages were maintained in a room with constant temperature 25 ± 1°C, humidity 60 to 70% and photoperiod 16:8 hour light: dark. Plastic trays (26x51 cm wide 4x4x5.5 cm^3 dimension) tightly covered with fine muslin cloth for aeration was used for larval rearing. Two days before treatment application larvae were allowed to feed on 4 weeks old tomato leaves provided in discs so that the larvae may acclimatize with natural diet again. The newly emerged pupae were removed from the diet sexed and moved to empty glass vials sealed with cotton wool and placed in the incubator maintained at 24±3°C, 70±5% RH and L14:D10 till the adult emergence. The newly developed adult moths and their sex were recorded daily and transferred to boxes to get eggs for the development of next progeny.

Bioassay Fungi

Fungal isolate (*Metarhizium robertsii* strain name Elateridae1) was derived from the collection of the Benaki Phytopathological Institute. 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 fromthe 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 solutionwith a magnetic stirrer for 5min. 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.

Experimental Protocol

The 2nd and 4th instar larvae, pupation, adult emergence and egg eclosion was determined by treating *H. armigera* larvae with a single dose rate of *B. thuringiensis* (Bt: 1 ml/L) and three of *M. robertsii* (*Mr*1: $1x10^3$, *Mr*2: $1x10^5$, *Mr*3: $1x10^7$ conidia/ml) individually and in their respective combinations (Bt+*Mr*1, Bt+*Mr*2 and Bt+*Mr*3). *B. thuringiensis* was applied by dipping the tomato leaf disc (3 cm each) from 4 weeks old seedlings into bacterium solution at dose rate of 1 µl/L for 3 minutes in a petri dish. Treated maize leaves were offered to the larvae in a sterilized petri dish for 48 hours. Larvae were then offered with the fresh untreated tomato leaves until they pupated or died. Before treating with *B. thuringiensis*, maize leaves were washed in a solution of commercial bleach (3% sodium hypochlorite) for 2-3 minutes to remove any kind of debris or disease causing agent and then allowed to dry. However *M. robertsii* was applied following larval immersion method (Ma *et al*., 2008). To check the synergistic effect of *M. robertsii* and *B. thuringiensis*, larvae were exposed to *B. thuringiensis* by feeding them on treated maize leaves for 48 hours and then dipped in fungal suspension for 10 seconds. The larvae were then allowed to feed on fresh untreated maize leaves until they pupated or died. Larval pupation rate and egg eclosion were recorded. The experiment was carried out in completely randomized design using 10 larvae of each 2nd and 4th instars per replicate and the bioassay was repeated three times. The determined number of each larval instar was collected from the culture.

Mathematical estimation

The interaction between the 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 $x^2 < 3.84$, the ratio is defined as additive, $x^2 > 3.84$ and the mortality observed higher than expected, the relationship is defined as synergistic. On the contrary, if $x^2 > 3.84$ and the mortality observed less than expected, the relationship is defined as competitive.

Statistical analysis

Data were normalized and then analyzed pupation, adult emergence, and egg eclosion with one-way ANOVA, using the general linear model of the SPSS (SPSS Inc., IL, USA, version 22) (IBM 2013). In case of significant F values, means were compared using the Bonferroni test.

3. Results

A significantly lower pupation and adult emergence was recorded in tested larval instars of *H. armigera* in the treatments where mortality rate was high (Table 1). In control treatment, 98.61 and 96.03% larvae of 2^{nd} and 4^{th} instar larvae successfully transformed into pupae. Unlikely, minimum pupation was observed in case of both 2nd (0%) and 4th (0%) instars when highest concentration of *M. robertsii* (1×10^7conidia/ml) and *B. thurengiensis* were applied simultaneously (*Mr*3 x Bt) (Table 1). Eggs laid by the adults from all the treatments were investigated for eclosion. Besides untreated check maximum egg eclosion was recorded in those eggs that were deposited by female adults developed from 2^{nd} and 4^{th} instar larvae treated with *M. robertsii* alone at a concentration of 1×10^3 conidia/ml and exhibited 84.00 and 89.29% eclosion respectively. However in case of combined application of *B. thuringiensis* and *Mr*3 (1×10^7 conidia/ml), there was no egg eclosion as no adult emerged from this treatment. Egg eclosion was also found in a direct relation to the adults emerged in all the treatments (Table 1). Our results showed that the combination of two pathogenic microorganisms increased the mortality of H. armigera larvaeduring the course of time. The results of the combined treatments showed a distinct interaction between pathogens after 15d as follows: For 2nd larval instars after 8 days: In two of the combinations interaction of the pathogens was additive and in one combination the interaction was synergistic (Table 1). For 4th larval instars after 8 days: In two of the combinations interaction of the pathogens was additive and in one combination the interaction was synergistic (Table 1).

4. Discussion

The current study was conducted to assess the effect of *M. robertsii* and *B. thuringiensis* against 2^{nd} and 4^{th} larval instars of *H. armigera* both individually and synergistically. Both larval stages showed varied mortality responses to various concentrations of fungi alone and in combination with *B. thuringiensis* used in this study.

In the present findings, virulency of *B. thuringiensis* toxin was in inverse relation with the growth and development of *H. armigera* larvae. Similar downfall in the efficacy of Bt against *H. zea* with the growth of the larvae was reported by (Herbert and Harper, 1985). Similarly, after 96 hours of *B. thuringiensis* application,

a mortality of 40-98% in 2nd instars compared with 52% in 3rd instar of the Colorado potato beetle was reported by (Zehnder and Gelernter, 1989). Moreover, Lacey *et al*. (1999) reported good to excellent control of Colorado potato beetle with the application of low to high concentrations (1.17 and 7.0 l ha^{-1}) of *B. thuringiensis* respectively. Enzymatic activity is responsible for differences in mortality of different larval instars. It has been stated that the action of detoxification enzymes changes significantly within and among different developmental stages. This action is minor in egg stage, amplifies with each nymphal or larval stage and then again reduces to zero at pupal stage (Ahmad, 1986; Mullin, 1988).

The results indicate clearly that a significantly higher larval mortality was observed in both instars larvae when *M. robertsii* was synergized with *B. thuringiensis.* These outcomes are in harmony with the findings of Lacey *et al*. (1999), who also reported the higher larval mortality of Colorado potato beetle in the plots treated with the combined use of *B. thuringiensis* and entomopathogenic fungi; while lowest mortality was recorded in untreated checks. Similarly combined application of *B. bassiana* and *B. thuringiensis* caused significant larval mortality of *Leptinotarsa decemlineata* compared with their individual applications (Wraight and Ramos, 2005). Similarly, Lewis *et al*. (1996) also concluded that integrated use of *B. thuringiensis* and *B. bassiana* enhanced the larval mortality of *Ostrinia nubilalis.*

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 enhance mycosis development. In this case, the effect of accelerated mortality is similar to starvation of insects (Mantzoukas *et al.* 2013). The synergistic action of entomopathogenic fungi and bacteria was further supported by Gao *et al.* (2012) who found that starvation stress imposed by Bt intoxication may cause negative effects on host immunity and physiology. This arrested nutrition by Bt (Kryukov *et al.*, 2009) further made the host more vulnerable to fungal spores which expedite the killing process. Furthermore, the starvation stress also enhanced the inter-molt period and this might be the possible cause for improved vulnerability of the larvae of *L. decemlineata* (Furlong and Groden, 2003). Moreover Lawo *et al.* (2008) found subleathal Cry2Aa intoxication of Bt against *H. armigera* increase the efficacy of *M. anisopliae.* Similarly

combined application of both bio control agents showed significant effect on the following developmental stages of either instar larvae with no or little pupation or egg eclosion.

Our study gives a hint that the entomopathogenic fungi and the *B. thuringiensis* can be used as potential biocontrol agents especially when applied synergistically for the management of maize stem borer. Moreover pest activity will require intensive scouting to determine the correct application timings of control agents. Different larval instars responded differently to biocontrol agents require elucidations of the characters responsible for the behavioural and physiological responses open up new avenues for further investigation. However the mortality obtained in the current laboratory bioassays may not predict the correct field mortality and there is need to conduct extensive field studies to check the combined efficacy of *M. robertsii* and *B. thuringienis* so that to develop and corroborate the successful integrated pest management solutions against *H. armigera*.

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Table 1. Mean effect of *Metarhizium robertsii* (*Mr* and *Bacillus thuringiensis* (Bt) alone and in combination on pupation, egg eclosion and treatment interaction of 2nd and 4th larval instars of H. armigera. Means sharing with same lower case letters are not significantly different from each other at 5% significance level.

Treatment	**Pupation (%)**		**Egg eclosion (%)**		**Interaction (1df, P= 0.05)**	
	2nd Instar	**4th Instar**	**2nd Instar**	**4th Instar**	**2nd Instar**	**4th Instar**
***Mr*1** *	82.00ab	74.16ab	84.00b	89.29a		
***Mr*2** **	55.27bc	67.21bc	70.66c	76.69b		
***Mr*3** ***	41.67de	54.17de	46.69e	53.35d		
Bt †	54.17cd	63.88cd	58.52d	58.39c		
***Ma*1 x Bt**	31.94ef	30.27e	27.31f	36.68e	A	A
***Ma*2 x Bt**	15.05fg	10.61f	13.46g	10.00f	A	A
***Ma*3 x Bt**	0.00g	0.00g	0.00h	0.00g	S	S
Control	98.61a	96.03a	99.24a	99.32a		

* Mr1: 103 conidia/ml

** Mr2:105 conidia/ml

*** Mr3: 107 conidia/ml

Bt †: 1 µl/L.

C= Competitive, A=Additive, S= Synergistic