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Effectiveness of Sublethal Doses of *Metarhizium robertsii* with *Bacillus thuringiensis*, Individual and Combined Treatment Against the Tomato Leafminer In Vitro

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Abstract

The objective of the present study was to evaluate how susceptible Tuta absoluta Meyrick (Lepidoptera: Gelechiidae) populations are to Metarhizium robertsii and Bacillus thuringiensis (Bt) in laboratory conditions. Larvae of T. absoluta were tested both separately and in combination with B. thuringiensis (Bt) at 1.5 µl/L, and with M. robertsii at 1 × 10^4, 1 × 10^5 and 1 × 10^6 spores/ml. Maximum mortality of 4th instar larvae was documented for M. robertsii (1 × 10^6 spores/ml) and B. thuringiensis (1.5 µl/L) were combined. In comparison with the controls, larval mortality, pupation and adult emergence rates of larvae of both instars were significantly lower when M. robertsii (1 × 10^6 spores/ml) and B. thuringiensis (1.5 µl/L) were applied in combination. Our results suggest that the combination of entomopathogenic fungi and the insecticidal protein of B. thuringiensis can be exploited as potential biocontrol agents against T. absoluta.

Keywords: *Bacillus thuringiensis*, biological control, *Tuta absoluta*, *Metarhizium robertsii*, tomato

Introduction

Tomato (*Solanum lycopersicum* L.), of the Solanaceae family, occupies a significant niche in the popular vegetable crops in Greece. The usual control strategy against *Tuta absoluta* (Gelechiidae) lies in the application of synthetic insecticides which has been steadily on the increase. However, chemical control entails the development of pest resistance to insecticides, as well as adverse side effects for natural enemies, the environment and humans. Considering these challenges, scientific research is geared towards the development of alternative control methods and other approaches within Integrated Pest Management [1–6].

In the toolbox against *T. absoluta* [7–9], biological control is considered as a cost effective and environmentally safe strategy against this pest in Europe [6]. The use of microorganisms can be used in place of chemical insecticides, as microorganisms are marked by environmentally safe activity and pest selectivity. They can be used either in

themselves or in conjunction with other pest control methods. Moreover, because they are very target specific, they do not harm beneficial fauna. The entomopathogenic fungus *Metarhizium robertsii* and the bacterium *Bacillus thuringiensis* (Bt) hold an important place in crop protection for their effectivity against pests and their environmentally safe profile [10].

The entomopathogenic fungus *M. robertsii* is an alternative tool in the management of lepidopteran pests [5, 6]. It mainly infects the host upon direct contact through conidia, blastospores, hyphae and other such propagules. Moreover, secondary infection is possible as spores can be transmitted from mycosed cadavers [11]. Mycospores latch on and penetrate the cuticle upon contact, without the need to be ingested first. They then grow inside the host, producing various toxins which inflict death upon the host pest [10, 12]. *B. thuringiensis* (Bt) is a gram-positive, rod-shaped bacterium which inhabits the soil and produces the so-called Cry and Cyt toxins, both while it sporulates and grows [13, 14]. Cry toxins paralyze the mouthparts and gut of the host which consequently ceases to eat. The host eventually stops feeding and dies [14]. The specific range of action of these toxic proteins against insects [15, 16] renders them excellent candidates for application in a wide array of sensitive crops where chemicals could potentially have severe effects [17]. Literature reports that intoxication with *B. thuringiensis* commercial formulations enhances the efficacy of entomopathogenic fungi against lepidopteran pests when applied in an integrated mode [18, 19]. Research shows that the joint action of *B. thuringiensis* and *M. robertsii* can have positive effect as combined which could be attributed to the starvation of larvae [20]. Keeping in sight the promise that these biological control agents show, we designed this experiment to study the individual and combined effects of *M. robertsii* and *B. thuringiensis* on the mortality of *T. absoluta* 2nd and 4th instar larvae.

Materials and Methods

We used 6-week-old tomato plants (*Solanum lycopersicum* L.). The plants were grown in controlled chambers (24 ± 1°C, r.h. 65%, photoperiod 16L: 8D) and supplied daily with a nutrient solution. The initial batch of *T. absoluta* larvae was collected from tomato cultivations in Mirtia, Ilia, Greece (37.702267, 21.359392) (Figure 1), in June 2017, for mass culturing in the laboratory. For our experiment, the insects were reared on tomato plants in growth chambers (24 ± 1°C, r.h. 70%, photoperiod 16L: 8D), inside cages (55 × 75 × 80 cm). During the experiment, *T. absoluta* larvae were reared on tomato leaves at 22.8–24.0°C, 12 h photophase and r.h. 82–100%.

Figure 1: Experimental field at Ilia, Greece where *T. absoluta* larvae were collected.

The fungal isolates of *M. robertsii*, strain MetAr2 (Figure 2) from different hosts in various regions in Western Greece. In order to prepare the suspensions appropriately, the isolates were grown in Parafilm®-sealed 9cm Ø Petri dishes containing Sabouraud dextrose agar, in the dark, at 25°C ± 1 for a total of 15 days. For each bioassay dose, fresh conidia were scraped off the Petri dish after 15 days, with a sterile loop. They were transferred to a 500 ml capacity glass beaker with 50 ml of distilled water and 0.05% Tergitol® NP9. The conidial suspension was placed on layers of sterile cloth and was prepared by mixing the solution with a magnetic stirrer for 5 min [2, 4–6, 21]. The desired conidial suspensions were achieved with a Neubauer hemocytometer and a phase contrast microscope at 400X magnification.

Figure 2: Laboratory culture of *M. robertsii*.

For the bacterial treatments, we used BactoSpeine® which is based on *B. thuringiensis* subsp. *kurstaki* (Hellafarm A.E, Greece). The insecticide has a 32.000 IU/mg potency and it is manufactured as granules and wet table powder (WP). The aqueous suspension of each dose was produced by mixing the powder with water in a sterile Erlenmeyer flask (100ml) at the desired concentrations. The solutions were stirred with a magnetic stirrer for 3 min.

Bioassay

We treated *T. absoluta* larvae with a single dose of *B. thuringiensis* (Bt: 1.5 µl/L) and three doses of *M. robertsii* (Mr1: 1×10^4, Mr2: 1×10^5, Mr3: 1×10^6 conidia/ml), both individually and combined (Bt + Mr1, Bt + Mr2 and Bt+ Mr3), to assess their mortality effect of 2nd and 4th instars. We dipped 3 cm tomato leaf fragments, from 4-week-old seedlings into the 1.5 µl/L *B. thuringiensis* solution for 3 min. The *B. thuringiensis* treated tomato leaf fragments were then left with the larvae as food, in a sterile Petri dish for 48 h. Preceding treatment with *B. thuringiensis*, tomato leaves had been cleared with a commercial bleach solution (3% sodium hypochlorite) for 2–3 min. This was done to clear them of any debris or disease-inducing factors. We then allowed time for them to dry.

M. robertsii was applied following Ma et al. [22] larval immersion method. To investigate the synergistic interaction between *M. robertsii* and *B. thuringiensis*, larvae were first supplied with *B. thuringiensis* treated tomato leaves for 48 h to feed. Larvae were then immersed in the fungal suspension for 10 sec [23]. From then onward, larvae were supplied with untreated tomato leaves. The rates of larval mortality, pupation rate, adult emergence, mycosis and sporulation were recorded. The experiment was carried out in a completely randomized experimental fashion using five larvae of the 2nd instar and five larvae of the 4th instar per replicate. The bioassay was repeated ten times. We observed each Petri dish for 7 days after the treatment. Observation consisted of placing their contents on sterile white paper to single out dead individuals. Dead individuals were collected, and they were handled in the following order: they were placed in 95% ethanol for 1 min; they were washed in distilled water for 5 min; once dry, they were placed on moistened filter paper. Cadavers were maintained in the dark at 25°C for 5–7 days. The ones which developed signs of fungal infection (growth of hyphae) were noted as infected.

Statistical analysis

Abbott's formula [24] was used to calculate corrected percent mortality. The values had been arcsine transformed before the analysis. Larval mortality, pupation, adult emergence, mycosis and sporulation data was IBM-processed (SPSS Inc., IL, USA, version 23.0.) (SAS Institute 2013) with two-way variance analysis (ANOVA). We used the Bonferroni test to separate means at a significance of 5% [2]. The percentages of sporulating cadavers were compared between doses using the t-test of the SPSS.

Results

Larval mortality of *T. absoluta*

Significant differences were recorded between treatments and the days of the experiment as factors, in relation to the dependent variable of mortality (Table 1). An upward trend in the mortality of *T. absoluta* larvae was observed at the increased dose and in the combined use of *M. robertsii* and *B. thuringiensis*. We recorded a significant mortality of 100% and 96.95% in 2nd and 4th instars respectively, when *M. robertsii* (1×10^6 conidia/ml) was combined with *B. thuringiensis* (0.25 µl/L). The lowest mortality in 2nd (8.08%) and 4th instars (7.12%) was recorded when treated with Mr1 (at 1×10^4 *M. robertsii* conidia/ml) and it was significantly different from the combined applications of *M. robertsii* and *B. thuringiensis*. Similarly, the individual application of *B. thuringiensis* caused a significantly lower mortality to 2nd (21.68%) and 4th (10.98%) instars, compared with the combined application of both entomopathogenic agents. The highest mortality was then followed by a mortality of 70.18% and 61.16% in 2nd and 4th instars, respectively, in the joint treatment with Mr2 and Bt. No mortality was observed for any control. Finally, 2nd instars were found to be more susceptible than 4th instars; in fact, mortality turned out to be inversely related to the larval developmental stage in all tested treatments.

Dependent variable: mortality					
	Sum of squares	**df**	**Mean square**	**F**	**Sig.**
Corrected model	1.788	299	0.006	1.903	0
Intercept	1.550	1	1.550	493.201	0
Days	0.688	14	0.049	15.641	0
Treatment	0.089	9	0.010	3.158	0.001
Days*treatment	0.419	126	0.003	1.057	0.332
Error	1.885	600	0.003		
Total	5.223	900			
Corrected total	3.673	899			

Table 1: Two-way ANOVA post-hoc Bonferroni test with mortality as variable.

Fungal growth on cadavers of *T. absoluta*

We observed a high rate of mycosis on cadavers following treatment of *T. absoluta* larvae with *M. robertsii*, especially at 1×10^6 conidia/ml ($t = 12.009$, $df = 5$, $P < 0.001$).

Discussion

The objective of the present experiment was to investigate how *M. robertsii* and *B. thuringiensis* work against 2nd and 4th larval instars of *T. absoluta*, both individually and synergistically. Both larval stages showed a variation in mortality rates post application of the different fungal concentrations, either used separately or in combination with *B. thuringiensis*. Entomopathogenic fungi in themselves appear very promising in the suppression of lepidopterous pests [4–6, 25], which is corroborated by the current findings in which *M. robertsii* caused significant mortality (> 40%) in both instars, at the highest dose. This was especially true for 2nd instars whose mortality surpassed 50%; this may be accounted for by the fact that fungi suppress the neonate *T. absoluta* larvae which feed externally on tomato leaves. Older instars, by contrast, which feed their way into the leaves and exist cryptically, may not be similarly infected by fungal applications. Mortality of older instars can be achieved when they are infected by mycosed cadavers inside the stem [26]. Nguyen et al. [27] reported effective action of *M. robertsii* against the various larval instars of *H. armigera*. In our experiment, a dose-dependent increase in mortality is in line with the study of Sasidharan et al. [28] who reported as far as 100% of larval mortality of *I. quadrinotata* Walker when treated with higher doses of *B. bassiana*, in contrast to 66.7% of larval mortality when treated with lower fungal concentrations.

A similar decrease in the efficacy of *B. thurigiensis* against *H. zea* as larvae grew was reported by Herbert et al. [29]. Similarly, 96 h after the application of *B. thuringiensis*, Zehnder et al. [30] reported a 40–98% mortality of 2nd instars of the Colorado potato beetle in contrast to 52% of mortality of 3rd instars. Moreover, Lacey et al. [31] noted that the control of the Colorado potato beetle was good at low *B. thuringiensis* concentrations and excellent at high *B.*

thuringiensis concentrations. The activity of enzymes explains differences in the mortality of different larval instars as the action of detoxification enzymes has been measured to vary significantly within and between different developmental stages. The egg stage exhibits minor enzymatic activity, the nymphal or larval stage is marked by an upsurge in enzymatic activity while the pupal stage presents no enzymatic activity whatsoever [32].

The results clearly show that a much higher larval mortality occurred in both larval instars when *M. robertsii* was synergized with *B. thuringiensis*. Our results are in sync with Lacey et al. [31] who also found that the mortality of the Colorado potato beetle was at its highest with the combination of *B. thuringiensis* and entomopathogenic fungi, and at its lowest in the controls. In a similar vein, when *B. bassiana* and *B. thuringiensis* were used together, they caused significant rates of larval mortality of *Leptinotarsa decemlineata*, as opposed to their individual applications [33]. Lewis et al. [34] also concluded that the integrated use of *B. thuringiensis* and *B. bassiana* reinforced the mortality rate of *Ostrinia nubilalis*. The synergy between entomopathogenic fungi and bacteria was also explored by Gao et al. [18] who concluded that the Bt-induced starvation of the host may disrupt its immunity and physiology. Moreover, the cessation of nutrition brought about by Bt intoxication [20] enhanced host vulnerability to fungal spores, thus expediting the killing effect. In addition, the starvation-related stress prolongs the inter-molt period which could account for the increased vulnerability of *Leptinotarsa decemlineata* larvae [35]. Lastly, Lawo et al. [36] found that the sublethal Bt Cry2Aa intoxication of *H. armigera* increases the efficacy of *M. robertsii*.

Our study corroborates the synergy of entomopathogenic fungi with *B. thuringiensis*. This synergy could be exploited in biological control to reinforce the management of the tomato leafminer. The activity of pests, however, must be closely monitored to determine the appropriate times for the application of these agents. Moreover, further research is required into the reasons for the variance in the behavioral and physiological responses of pests at different larval stages. As laboratory mortality cannot accurately predict field mortality, extensive field studies are required to assess the combined efficacy of *M. robertsii* and *B. thuringiensis* against *T. absoluta*.

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