Compatibility among entomopathogenic hyphocreales and two beneficial insects used to control Trialeurodes vaporariorum (Hemiptera : Aleurodidae) in Mediterranean greenhouses

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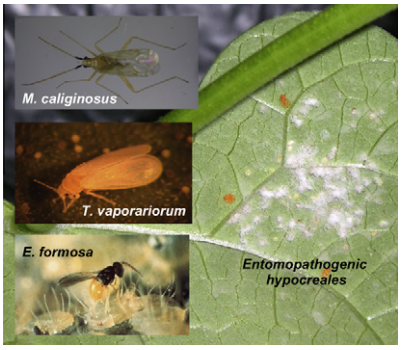
Graphical abstract

Compatibility among entomopathogenic hyphocreales and two beneficial insects used to control *Trialeurodes vaporariorum* (Hemiptera: Aleurodidae) in Mediterranean greenhouses pp xxx–xxx

Faten Hamdi, Jacques Fargues, Gilles Ridray, Benoît Jeannequin, Olivier Bonato *



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Highlights

▶ *Beauveria bassiana* and *Leucanicillium muscarium*-based formulations and *Encarsia formosa* kill *Trialeurodes vaporariorum* larvae either separately or in association. ▶ Efficacy of parasitization was higher in larvae treated with *B. bassiana* and exposed to *E. formosa.* ▶ Third-instar larvae of *T. vaporariorum* showed a low susceptibility to tested fungi. ▶ Second-instar larvae of *Macrolophus caliginosus* were not susceptible to *L. muscarium* and *B. bassiana* formulations. ▶ *M. caliginosus* populations treated with fungi in greenhouse conditions were not Q1 significantly affected.

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Compatibility among entomopathogenic hyphocreales and two beneficial insects used to control *Trialeurodes vaporariorum* (Hemiptera: Aleurodidae) in Mediterranean greenhouses

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ABSTRACT

The effect of the combined use of *Encarsia formosa* or *Macrolophus caliginosus* and one of three marketed mycoinsecticides, Mycotal® (*Leucanicillium muscarium*-based), Naturalis-L™ (*Beauveria bassiana*-based) and PreFeRal® (*Isaria fumosorosea*-based), on the control of the whitefly, *Trialeurodes vaporariorum,* was studied under laboratory and greenhouse conditions. The results of both types of tests, the bioassays and the greenhouse trials, for all combinations of *E. Formosa* with each of the three mycoinsecticides showed that the total mortality of larval populations of *T. vaporariorum* was not affected. The mortality of *T. vaporariorum* larvae treated in the second instar revealed the capacity for both *B. bassiana*- and *L. muscarium*-based formulations and *E. formosa* to kill the host either separately or in association. Because of its higher pathogenic activity (under our test conditions), *L. muscarium* provoked a large proportion of mycoses in larvae exposed to parasitization. In contrast, the efficacy of parasitization was higher in larvae treated with *B. bassiana* and exposed to *E. formosa* because of a lower pathogenic activity of the fungus. Bioassays carried out with third-instar larvae of *T. vaporariorum* showed a low susceptibility to both tested fungi. Consequently, mortalities recorded in larvae subjected to the combined treatments by consecutive exposures or at 2–4 days post-parasitization were mainly caused by the development of the parasitoid. Greenhouse trials showed that fungus-induced mortality of *T. vaporariorum* in plants treated with *L. muscarium*, *I. fumosorosea*, and *B. Bassiana* was significant compare to control. *L. muscarium, B. bassiana* and *I. fumosorosea* killed young whitefly larvae and limited parasitization to 10% or less. Second-instar larvae of *M. caliginosus* were not susceptible to *L. muscarium* and *B. bassiana* formulations with any mode of contamination: direct spraying of larvae, spraying on the foliar substrate or by contaminated *T. vaporariorum* prey. In greenhouse trials, *M. caliginosus* populations treated with fungi were not significantly affected compared to controls.

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1. Introduction

The greenhouse whitefly, *Trialeurodes vaporariorum* Westwood, is a polyphagous and cosmopolitan species (van Lenteren and Noldus, 1990). It still remains the primary insect pest of many greenhouse crops, including tomato. Resistance to chemical insecticides and the negative impacts of these insecticides in integrated control programs have encouraged the development of alternative control. Interest in natural enemies increased with the use of whitefly parasitoids at the start of the 1970s (van Lenteren et al., 1996), and the technique was rapidly extended to predatory bugs of the genera *Macrolophus* and *Dicyphus* (Onillon, 1990). The parasitoid *Encarsia formosa* Gahan is available commercially for greenhouse crops and serves as an effective control through only a few introductions to European greenhouse tomatoes (van Lenteren, 1992; van Lenteren and de Ponti, 1990). Among all predators, *Macrolophus caliginosus* Wagner, the most abundant species, is notable for its contribution to the control of *T. vaporariorum* (Alomar et al., 1994; Pasini et al., 1998). This natural enemy is frequently encountered at the end of spring in the northwestern Mediterranean basin in protected crops when no pesticide has been applied (Castañé et al., 1997, 2004). Its spontaneous establishment, geographical distribution, biological attributes and status as a natural enemy ensure this mirid a central role in biological control programs. Fauvel et al. (1987) confirmed that *M. caliginosus* is an active predator on immature stages of the Greenhouse Whitefly, *T. vaporariorum.* Since the beginning of the 1990s, *M. caliginosus* has been distributed by companies specializing in the marketing of natural enemies of pests.

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Bioinsecticides based on entomopathogenic hypocreales (Ascomycota) were deemed promising for whitefly management because they were the only ones able to infect plant-sucking insects through penetration of the cuticle (Fransen, 1990). Various authors have demonstrated the efficacy of the hyphomycetes *Beauveria bassiana* (Balsamo) Vuillemin, *Isaria fumosorosea* Wize (formerly *Paecilomyces fumosoroseus*) and *Lecanicillium muscarium* (Petch) Zare and Gams (formerly *Verticillium lecanii*) as mycoinsecticides for controlling whiteflies (Osborne and Landa, 1992; Lacey et al., 1996).

Possible interactions between these different types of biological agents have the potential to change the global regulation of the targeted populations (Brooks, 1993). Depending on the protagonists, their interactions may be detrimental, inconsequential or even mutually beneficial (Brooks, 1993). Some aspects of fungus-parasitoid interactions have been studied, mainly in aphid hosts (Milner et al., 1984; Powell et al., 1986; Brobyn et al., 1988; Poprawskti et al., 1992; Mesquita et al., 1997; Mesquita and Lacey, 2001) and, to a lesser extent, in whitefly hosts (Fransen and van Lenteren, 1994). Despite these studies, there is a relative lack of data on the susceptibility of parasitoids and predators to entomopathogenic fungi used as mycoinsecticides under laboratory and field conditions. Because of the expansion of biological pest management in the Mediterranean greenhouse industry, the effect of mycoinsecticides on the activity of other commonly used biocontrol agents has become an important issue. The present work was conducted to study the effects of fungus-based products used for whitefly control in Mediterranean greenhouses, alone and in combination with the biocontrol agents *E. formosa* and *M. caliginosus*, which are frequently used in both inoculative and inundative biological control strategies (Albajes and Alomar, 1999; Gerling et al., 2001; Gabarra et al., 2004).

2. Material and methods

2.1. Fungi

Three mycoinsecticides were selected for their efficacy against second-instar larvae of *T. vaporariorum*, as shown in trials conducted in tomato greenhouses under Mediterranean conditions (Fargues et al., 2003, 2005; Vidal et al., 2003). However, the goal of this study was not to compare their relative efficacy but to investigate the interactions between fungus-induced disease and natural enemies. The mycoinsecticides were applied at recommended dosages. For greenhouse crop protection, manufacturers recommend 3–5 successive applications of 1000–3000 l of water suspension of formulated fungus at 3–7 day intervals according to both pest pressure and canopy conditions. The *B. bassiana*-based product, Naturalis-L™ (Troy Biosciences Inc., Phoenix, AZ 85009, USA), was used in an emulsifiable vegetable oil formulation. I. It was initially concentrated by the manufacturer at 1×10^8 conidia per ml with a label claim of 2.3 x 107 viable conidia ml^{-1}. Naturalis-L was used at the recommended dosage of 0.3% (V/V). The *L. muscarium*-based product, Mycotal® (Koppert B.V., Berkel en Rodenrijs, The Netherlands) consisted of a wettable powder (WP) with a label claim of 1×10^{10} viable spores $gram^{-1}$ used in combination with 0.25% emulsifiable oil with spreaders (Koppert Oil Formulation as KOF). Mycotal was used at the recommended dosage of 0.1% (W/V). The *I. fumosorosea*-based product, PreFeRal® (Biobest, Westerlo, Belgium), was used as a granular formulation with a label claim of $1 \times 10^{\varsigma}$ CFU $gram^{-1}$ at a rate of 0.1% (W/V). Immediately prior to experiments (bioassays and greenhouse trials), the viability of each batch of formulated fungal propagules was recorded *in vitro* by counting colony forming units (CFU) according to Fargues et al. (1994, 2003) and Vidal et al. (2003). The viability of propagules exceeded 90% excepted in one batch of *B. bassiana* formulated inoculum with a viability of 86%. Fungus-induced mortality caused by this batch for one series of assays did not differ from that observed in replicates.

2.2. Insects

Whiteflies used in the bioassays were obtained from a colony maintained at the Institut National de la Recherche Agronomique (INRA) laboratory in Antibes. They were reared in screened cages (50 × 50 × 50 cm) containing young green bean plants (*Phaseolus vulgaris* L., c.v. Contender, Oxadis, La Verpillière, France) at daytime temperatures of 22 ± 1 °C and nighttime temperatures of 18 ± 1 °C and a photoperiod of 16:8 h (L:D). Once seedlings were at the 3- to 4-leaf stage, they were transplanted into 1.5-l pots filled with soil. The central leaf of the true first leaves of 5-week-old plants was selected for artificial infestation. Twenty young whitefly adults [sex-ratio of ≈1:1] were placed inside clip-cages (7 cm^3) on the undersides of the host-plant leaves. Whiteflies were given the opportunity to lay eggs for 24 h. Within 15 days, the undersides of the green bean leaves were infested with mostly second-instar larvae.

M. caliginosus larvae used in the experiments were mass-reared at the Centre de Biologie pour la Gestion des Populations (CBGP/INRA-IRD) from stock originating from both Koppert BV (Mirical®, Berkel en Rodenrjs, The Netherlands) and the GIE Lacroix (Runavel, Guipavas, France). Insects were reared on tobacco (*Nicotiana tabacum*) plants, fed on eggs of *Ephestia küniella* Zeller (Lepidoptera: Pyralidae) provided by Koppert (Entofood®) and maintained at 22 ± 1 °C, 50–65 RH% and under a photoperiod of 16:8 h (L:D). Homogeneous cohorts were obtained by sampling lots of mated females and then using eggs deposited over a period of 48 h. Under these conditions, one hundred females provided a homogenous cohort of ca. 300 s-instar larvae in 3 weeks.

E. formosa adults used in these experiments were obtained directly from Koppert as black pupae on cardboard cards. Black pupae were put in Petri dishes at 22 ± 1 °C under a photoperiod of 16:8 h (L:D). The emerging adults of *E. formosa* were kept in capsules with honey as food for 24 or 48 h. They were then put in clip cages on the patches with *T. vaporariorum* larvae and were given the opportunity to parasitize hosts for 48 h.

2.3. Bioassays

Mycoinsecticide treatments consisted of spraying fungal suspensions by using a spray tower apparatus originally described by Burgerjon (1956) (Luz and Fargues, 1998). This spray tower, dispensing known volumes of aqueous conidial suspensions at a regulated pressure, provides fine, non-drenching sprays with a deposit rate of 3 µl cm^{-2} when spraying 10 ml (Vidal et al., 1997, 2003). Inoculum dosages used in our bioassays were calculated to achieve the quantity recommended by the manufacturer for field application, which depends on the rate and coverage. In the spray device, fungal inocula consisted of ca 1.6×10^5 CFU of *B. bassiana* per cm^2 substrate surface and ca 3.9×10^4 CFU cm^{-2} of *L. muscarium*. These formulated fungal suspensions were sprayed on either potted whole green bean plants, contaminated with whiteflies or batches of 20 anaesthetized *M. caliginosus* larvae, or on potted whole tobacco plants for bugs. Controls were sprayed with distilled water because of the innocuity of the formulation ingredients tested in previous assays (~~Fargues et al., unpublished data~~). Q3

A bioassay was developed to test possible interactions between *E. formosa* and the mycoinsecticides based on *L. muscarium* and *B. bassiana* according three modalities. (a) In the first series, second-instar larvae of *T. vaporariorum* fixed on foliage of green bean plants were first treated with fungal inocula and then subjected to parasitization by *E. formosa* females in the first 2–4 days after the fungal

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contamination. (b) In the second series, third-instar larvae were sprayed with fungal inocula in the first 2–4 days following parasitization. (c) In the third series, third-instar larvae were subjected to parasitization immediately following the fungal contamination. The experimental device used for pathogen-parasitoid interactions consisted of exposing potted whole green bean plants, either whitefly-infested and fungus-treated or not, in experimental chambers at 20 °C and under a photoperiod of 16:8 h (L:D). These chambers consisted of an airtight plastic box (27 × 36 × 18 cm) containing three potted whole plants, the bases of which were put into plastic bags to prevent vapor from being released from the wet plant growth substrate (Vidal et al., 2003). The air was constantly circulated in the experimental chambers by a membrane air pump (720 l h^{-1}) after passage from a previous chamber (18 × 27 × 18 cm) containing a solute for regulating the air moisture (Vidal et al., 1997). Air exchange took 4–5 min to complete in the test chamber. Relative humidity was monitored within each test chamber using a probe attached to a data logger (21X Micrologger) (Vidal et al., 2003). In both series, air humidity was theoretically regulated at 75% RH with saturated NaCl. Because of plant transpiration, air humidity measured inside the test chambers ranged from 80–85% RH under dark conditions to 84–90% RH under light conditions.

A second type of bioassay was developed to test for possible effects of *L. muscarium* and *B. bassiana* treatments on the predatory bug, *M. caliginosus*. The fourth and fifth series of bioassays consisted of testing the susceptibility of second-instar larvae of *M. caliginosus* to both *B. bassiana* and *L. muscarium* formulations applied by direct spraying and by previous contamination of the foliar surface providing the substratum for predacious larvae, respectively. A sixth series of assays consisted of testing possible transmission of the fungal disease to second-instar larvae of *M. caliginosus* by feeding them fungus-contaminated larvae of *T. vaporariorum*. Prey subjected to predation for 72 h were previously fungus-contaminated, 24 h, 72 h, and 5 days before, respectively. After 24 and 72 h, fungus-treated *T. vaporariorum* larvae were still alive. At day 5 post fungus contamination, batches of larvae submitted to predation consisted in both surviving and freshly dead individuals. Just before introduction of *M. caliginosus*, *T. vaporariorum*-infested plants initially grouped per three were individually placed in experimental chambers to prevent passage bugs from one plant to another.

Individual nymphs of *T. vaporariorum* were monitored for mortality using a binocular microscope at a magnification of 10X. Assessments were made when most of the healthy larvae had developed into adult whiteflies (empty pupal cases). According to the bioassay design, dead larvae data consisted of parasitized larvae, predated larvae, and fungus-infected larvae. Larvae were considered to be parasitized when we observed the presence of black pupae or signs of emergence by an adult wasp. They were considered to be predated when the empty pupal cases did not present the characteristic exit hole caused by the emergence of an adult. Larvae were considered to be fungus-killed if they turned opaque and if mycelial outgrowths appeared when placed under high air humidity conditions in an incubator. Other dead larvae, considered to be the result of natural mortality, mainly consisted of desiccated larvae for which the cause of death was not detectable. Surviving whiteflies were defined as the surviving, apparently healthy larvae (which were in the fourth instar at the time of assessment) and empty pupal cases already abandoned by a whitefly adult. The developmental stage (second, third, or fourth instar) of each cadaver was also noted. Larvae of *M. caliginosus* were monitored 2 weeks after exposure to fungal inocula and fungus-contaminated prey.

2.4. Greenhouse experiments for fungus-E. formosa interactions

Experiments focused on the interactions between mycoinsecticide treatments and parasitization were conducted on tomato plants in a greenhouse at the INRA Experimental Research Unit located in Alenya (42° 38' N latitude, 2° 58' E longitude and 5 m altitude) in the south of France. An experimental, unheated plastic greenhouse (tunnel of 324 m^2, 31 m in length, 7 m in width, 3.2 m high) was used. The tunnel was planted with the tomato cultivar 'Petula' (Rijk Zwaan, La Vermède, Aramon, France) in a sandy loam soil.

On March 7, tomato plants were transplanted in two double rows in the center of the tunnel and two single rows along the two sides. The plants were spaced 90 cm apart between rows and 53 cm apart within rows. Experiments were performed between April 11 and May 9, 2005. The experimental design was a randomized complete block with three fungus-based formulations: Preferal, Naturalis-L, and Mycotal, and controls (water), replicated 4 times. Because of the immobility of whitefly larvae, tomato plants could be considered as units for mycoinsecticide treatments (Fargues et al., 2003, 2005). The tunnel was physically split (plastic wall) into 4 equal parcels. Within each of the four parcels, 16 plants located in both central double rows were used, with 4 plants randomly devoted to each of the 4 treatments, for a total of 48 treated plants in the greenhouse. The unit for sampling consisted of one artificially infested young leaf (third node from the growing tip) per selected plant. The 4 plants used with the same treatment within each pseudo-parcel consisted of 2 pairs of 2 plants. Each pair of plants consisted of plants directly across from one another in the double row, and pairs of treated plants were separated by two pairs of untreated guard plants.

Because of the low natural level of the whitefly population in situ, artificial infestations were carried out on randomly selected plants. Artificial infestation consisted of introducing 80 young whitefly adults (sex ratio 1:1) inside an organdy sleeve (300-*l*m mesh), wrapped around one young leaf of the previously selected plants. Whitefly adults were given the opportunity to lay eggs for 4 days, resulting in at least 30–60 eggs/leaf. After removing adults and sleeves, yellow sticky traps were installed near the infested leaves to catch escaped adults. When most of the individuals reached the second instar, the whitefly population was sprayed.

The *B. bassiana*-based product, Naturalis-L™ was used at 0.3% of emulsifiable vegetable oil formulation (V/V). The *L.muscarium*-based product, Mycotal was used at 0.1% (W/V) of wettable powder in combination with 0.25% emulsifiable oil. The *I. fumosorosea*-based product, PreFeRal® was used at 0.1% (W/V) of granular formulation. Just before greenhouse application, the viability of formulated fungal propagules was recorded *in vitro* by counting CFU obtained after 4–8 days of incubation on a glucose–yeast extract semi-synthetic medium. It exceeded 90% in all formulated batches. Applications were made using a single-nozzle, atomizing (air-assist), 15-l backpack sprayer (Berthoud–Exel GSA, Villefranche-sur-Saône, France) operating at a pressure of 2 bars. During spraying, the spray nozzle was directed at a right angle to the underleaf surface of each treated plant, making sure the undersides of the leaves were coated. Plants not selected for infestation were not sprayed. During the applications, non-target plants were protected from overspray by large plastic panels (2 m × 1.20 m). The control plants were sprayed with water. Applications were made on June 3 on populations of mostly second-instar larvae. A second treatment was repeated under the same conditions 5 days later on third-instar larvae. Because second-, third-, and fourth-instar larvae are sessile and attached to the leaf surface, mortality assessments were performed at the end of the trials. Sampling was conducted on the 19th day after fungal applications, when more than 80% of whitefly adults had emerged in the controls. Sampled leaves were individually placed in plastic bags (30 × 45 cm) and maintained at 4 °C until data were recorded, i.e. for 16–48 h. Individual larvae were monitored for mortality using a dissecting microscope at a magnification of 36X. Emerged whiteflies (empty pupal cases) and

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surviving larvae were counted. The number of parasitized larvae, predated larvae, and larvae dead of other causes were also counted. The developmental stage (second, third, or fourth instar) of each cadaver was noted. To avoid errors caused by double counting, each observed larva was marked with a permanent ink pen. The developmental stage of each cadaver was also noted.

2.5. Greenhouse experiments for L. muscarium–M. caliginosus interactions

The experiments investigating the impact of fungus as mycoinsecticide on *M. caliginosus* were conducted in two commercial plastic cold greenhouses (ogival tunnels of 324 m^2, 40.5 m length, 8 m width, and 4 m height) at the INRA Unit located in Alenya. Tomato plants of the cultivar 'Izabella' (Zeraim Iberica, Valencia, Spain) were infested on March 7, 2000 in a nursery with 6 *M. caliginosus* adults per plant. On March 20, plants at the 2- to 3-leaf stage were transplanted in three double rows in the center of the tunnel and in two single rows along the two sides. Plants were spaced 90 cm apart between rows and 53 cm apart within rows. Experiments were performed in April and May.

A factorial design was used. Each greenhouse was divided into 3 equal blocks of 8 pairs of plants (double rows) to account for the north–south climatic heterogeneity inside the tunnel. There were two guard plants in each row between the blocks. Plants along the northern and southern borders and plants in the single lateral rows were not used for sampling. Because of the immobility of whitefly larvae and the aggregative distribution of the larvae of *M. caliginosus*, tomato plants could be considered as units for sampling. Each block consisted of three pseudo-parcels of 36 plants (6 per row × 3 double rows). Parcels were selected randomly as controls and for fungus treatments. To avoid the undesirable projection of fungal inoculum, each parcel was isolated during spraying by using 1.90 m high and 3 m wide sections of plastic film.

Mycotal was used under the conditions recommended by the manufacturer, applied at a rate of 1 g of wettable powder per liter of water with 0.25% emulsifiable oil with spreaders. All applications were made using a single-nozzle, atomizing (air-assist), 15 l backpack sprayer (Berthoud–Exel GSA, Villefranche-sur-Saône, France) operating at a pressure of 4 bars and delivering 0.65 l min^{-1}. During spraying, the spray nozzle was directed at a right angle to the underleaf surface of each treated plant, making sure the undersides of the leaves were coated. The amount of water was estimated at 2000 l/h. The control plants were sprayed with water according to the results of previous assays showing no effect of formulation ingredients in greenhouse trials (Fargues et al., 2003). Treatments consisted of two successive applications at 7-day intervals.

2.6. Statistical analyses

Bioassays were carried out in a completely randomized experimental design. The entire set of bioassays was replicated four times. Both mortality and survival data expressed as proportions were arcsine-square root transformed, as suggested by Zar (1984), and then analyzed using one- or two-way analyses of variance (ANOVA) ($\alpha = 0.05$) followed by a comparison of the means using the Student–Newman–Keuls (SNK) multiple range test. Population density data, i.e., the total number of whitefly larvae (alive and dead) counted per sampled leaf, were analyzed without transformation. Analyses were performed using SigmaStat (SPSS, 1997) with either a one- or two-way ANOVA followed by the SNK multiple range test ($\alpha = 0.05$) (SAS Institute, 1989). Data are reported in both the text and tables as mean values ± standard error of the mean (SEM).

3. Results

3.1. Fungus-E. formosa interactions

The artificial whitefly infestation of green bean plants produced a relatively high larval density, ranging from 130 to 240 individuals per sampled leaf (Tables 1–3).

3.1.1. Experiment 1

The emergence of whitefly adults in bioassays of the parasitization by *E. formosa* of second-instar larvae of *T. vaporariorum* in the first 2–4 days after contamination with entomopathogenic fungal inocula varied significantly between treatments ($F = 40.13$; $df = 5.18$; $P < 0.001$) (Table 1). Emergence rates reached 90% in controls and were significantly lower in larvae subjected to fungal infection and parasitization. Adult emergence was very low in larvae subjected to parasitization by *E. formosa* without fungus (8%) and those subjected to a combination of *L. muscarium* (1%) and *B. bassiana* (10.7%). There were significant differences in fungus-induced mortality ($F = 14.10$; $df = 3, 12$; $P < 0.001$), parasitization ($F = 98.34$; $df = 2,9$; $P < 0001$), and parasitoid emergence ($F = 59.81$; $df = 2, 9$; $P < 0.001$). Fungus-induced mortality recorded in larvae treated with *L. muscarium* and *B. bassiana* formulations were 78.8% and 30.9%, respectively. The highest rates of parasitization (black pupae 89%) and parasitoid emergence (77.2%) were observed in larvae subjected to *E. formosa* without a fungal inoculum. When exposed to both *L. muscarium* inoculum and *E. formosa*, most larvae were fungus-killed (80.7%), and the other individuals were parasitized. In contrast, larvae exposed to both *B. bassiana* inoculum and *E. formosa* were mycosed at a rate of only 39.7% and

Table 1
Effect of parasitization by *Encarsia formosa* of second-instar *Trialeurodes vaporariorum* in the first 2–4 days after contamination with entomopathogenic fungal inocula: number of whitefly larvae per sampled leaf, emergence rate and mortality due to parasitization, to fungus infection or to other causes.

	No. Whiteflies/sampled leaf live & dead[a]	Emerged Whitefly adults[b]	Whitefly larval mortality[c]			Emerged parasitoids[b]
			Natural mortality	Fungus-induced Mortality	Parasitization (black pupae)	
Control	193.8 ± 20.6[a]	71.8 ± 3.0[a] (90.2%)	18.2 ± 3.0[a] (9.8%)	(0%)	(0%)	(0%)
E. formosa	143.3 ± 2.3[b]	16.4 ± 1.9[c] (8.0%)	8.2 ± 3.5[b] (2.0%)	(0%)	70.7 ± 2.2[a] (89.0%)	61.5 ± 2.6[a] (77.2%)
L. muscarium	161.0 ± 7.8[b]	26.9 ± 6.9[c] (20.5%)	2.7 ± 1.6[b] (0.2%)	62.6 ± 6.7[a] (78.8%)	(0%)	(0%)
E. formosa + *L. muscarium*	194.3 ± 17.1[a]	5.8 ± 3.6 d (1.0%)	1.5 ± 1.5[b] (0.1%)	63.9 ± 3.1[a] (80.7%)	24.4 ± 2.3[c] (17.1%)	21.0 ± 1.7[c] (12.9%)
B. bassiana	239.8 ± 25.7[a]	54.8 ± 3.6[b] (66.8%)	8.4 ± 1.7[b] (2.1%)	33.8 ± 3.1[b] (30.9%)	(0%)	(0%)
E. formosa + *B. bassiana*	213.0 ± 22.5[a]	19.1 ± 3.3[c] (10.7%)	1.6 ± 1.6[b] (0.1%)	39.1 ± 2.3[b] (39.7%)	44.1 ± 2.6[b] (48.4%)	41.4 ± 3.3[b] (43.7%)

[a,b,c] Means within a column followed by the same letter are not significantly different (ANOVA procedure; $\alpha = 0.05$; SNK test).
[a] Mean number of whitefly larvae recorded on each leaf (x ± sem).
[b] Mean survival (x ± SEM), expressed as angular value [arcsin $\sqrt{}$ (number of emerged adults/initial number of whiteflies)]. Emergence rates (%) in brackets.
[c] Mean larval mortality (x ± SEM), expressed as angular value [arcsin $\sqrt{}$ (number of dead larvae/initial number of whiteflies)]. Mortality rates (%) in brackets.

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Table 2
Effect of parasitization by *Encarsia formosa* of third-instar *Trialeurodes vaporariorum* just after contamination with entomopathogenic fungal inocula: number of whitefly larvae per sampled leaf, emergence rate and mortality due to parasitization, to fungus infection or to other causes.

	No. Whiteflies/sampled leaf live & dead[a]	Emerged Whitefly adults[b]	Whitefly larval mortality[c]			Emerged parasitoids[b]
			Natural mortality	Fungus-induced Mortality	Parasitization (black pupae)	
Control	202.5 ± 36.3[a]	65.6 ± 7.6[a] (83.0%)	24.4 ± 7.6[a] (17.0%)	(0%)	(0%)	(0%)
E. formosa	134.5 ± 20.6[a]	15.5 ± 2.4[b] (7.1%)	14.4 ± 5.3[a] (6.2%)	(0%)	66.6 ± 1.8[a] (84.2%)	56.3 ± 2.2[a] (69.2%)
L. muscarium	203.3 ± 11.2[a]	56.9 ± 6.2[a] (70.2%)	5.3 ± 2.0[a] (0.9%)	32.3 ± 6.2[a] (28.6%)	(0%)	(0%)
E. formosa + L. muscarium	168.3 ± 13.1[a]	9.2 ± 9.2[b] (2.5%)	14.1 ± 3.6[a] (6.0%)	30.9 ± 8.0[a] (26.4%)	47.9 ± 4.1[b] (55.0%)	42.5 ± 3.0[b] (45.7%)
B. bassiana	164.0 ± 29.0[a]	59.3 ± 7.8[a] (73.7%)	14.1 ± 8.0[a] (6.0%)	24.3 ± 2.9[a] (17.0%)	(0%)	(0%)
E. formosa + B. bassiana	191.5 ± 12.8[a]	12.6 ± 2.0[b] (4.8%)	5.4 ± 5.4[a] (0.9%)	27.8 ± 2.0[a] (21.8%)	56.7 ± 2.8[b] (69.9%)	47.6 ± 1.9[b] (54.6%)

[a,b,c] Means within a column followed by the same letter are not significantly different (ANOVA procedure; $\alpha = 0.05$; SNK test).
[a] Mean number of whitefly larvae recorded on each leaf ($x \pm$ sem).
[b] Mean survival ($x \pm$ SEM), expressed as angular value [arcsin $\sqrt{}$ (number of emerged adults/initial number of whiteflies)]. Emergence rates (%) in brackets.
[c] Mean larval mortality ($x \pm$ SEM), expressed as angular value [arcsin $\sqrt{}$ (number of dead larvae/initial number of whiteflies)]. Mortality rates (%) in brackets.

Table 3
Effect of entomopathogenic fungal contamination of third-instar *Trialeurodes vaporariorum* in the first 2–4 days following parasitization by *Encarsia formosa*: number of whitefly larvae per sampled leaf, emergence rate and mortality due to parasitization, to fungus infection or to other causes.

	No. Whiteflies/sampled leaf live & dead[a]	Emerged White fly adults[b]	Whitefly larval mortality[c]			Emerged parasitoids[b]
			Natural mortality	Fungus-induced Mortality	Parasitization (black pupae)	
Control	168. 0 ± 2.7[b]	78.3 ± 0.5[a] (95.9%)	11.7 ± 0.5[b] (4.1%)	(0%)	(0%)	(0%)
E. formosa	157.3 ± 12.2[b]	14.0 ± 2.4[b] (5.8%)	31.4 ± 3.0[a] (27.1%)	(0%)	54.6 ± 2.9[a] (66.5%)	54.6 ± 2.9[a] (66.5%)
L. muscarium	161.8 ± 20.8[b]	61.5 ± 13.7[a] (77.2%)	(0%)	28.50 ± 13.66[a] (22.8%)	(0%)	(0%)
E. formosa + L. muscarium	130.8 ± 2.5[b]	12.3 ± 1.4[b] (4.6%)	14.7 ± 4.0[b] (6.4%)	23.20 ± 10.74[a] (15.5%)	54.3 ± 5.7[a] (66.0%)	53.5 ± 6.2[a] (64.5%)
B. bassiana	228.5 ± 11.2[a]	73.3 ± 2.6[a] (91.8%)	6.7 ± 0.8 (1.4%)	15.10 ± 2.57[a] (6.8%)	(0%)	(0%)
E. formosa + B. bassiana	166.3 ± 11.2[b]	16.0 ± 4.2[b] (7.7%)	11.6 ± 0.8[b] (4.0%)	26.62 ± 9.81[a] (20.1%)	53.0 ± 8.8[a] (63.8%)	50.8 ± 7.4[a] (60.1%)

[a,b,c] Means within a column followed by the same letter are not significantly different (ANOVA procedure; $\alpha = 0.05$; SNK test).
[a] Mean number of whitefly larvae recorded on each leaf ($x \pm$ sem).
[b] Mean survival ($x \pm$ SEM), expressed as angular value [arcsin $\sqrt{}$ (number of emerged adults/initial number of whiteflies)]. Emergence rates (%) in brackets.
[c] Mean larval mortality ($x \pm$ SEM), expressed as angular value [arcsin $\sqrt{}$ (number of dead larvae/initial number of whiteflies)]. Mortality rates (%) in brackets.

parasitized at a rate of 48.4%. Natural mortality, without any specific symptom, remained at very low rates (⩽2.1%).

3.1.2. Experiment 2

The emergence of whitefly adults in bioassays of the parasitization by *E. formosa* of third-instar larvae of *T. vaporariorum* immediately following contamination with entomopathogenic fungal inocula depended on treatments ($F = 40.13$; $df = 5, 18$; $P < 0.001$) (Table 2). There were two distinct groups with respect to emergence rates: a high-emergence group consisting of controls (83%), *L. muscarium*-contaminated larvae (70.2%), and *B. bassiana*-contaminated larvae (73.7%); and a low-emergence group with larvae subjected to parasitization alone (7.1%) or combined with *L. muscarium* (2.5%) or *B. bassiana* (4.8%) (Table 2). Fungus-induced mortality recorded in larvae treated with *L. muscarium* and *B. bassiana* formulations alone (28.6% and 17.0%, respectively) or in combination with exposure to parasitization (26.4% and 21.8%, respectively) did not differ significantly ($F = 0.44$; $df = 3, 12$; $P = 0.729$). In contrast, both parasitization and parasitoid emergence rates were significantly higher in larvae exposed to *E. formosa* without fungus (84.2% and 69.2%, respectively) than in larvae subjected to parasitization in combination with either *L. muscarium* (55.0 and 45.7%, respectively) or *B. bassiana* (69.9% and 54.6%, respectively) ($F = 9.43$; $df = 2,9$; $P = 0.006$ and $F = 8.36$; $df = 2,9$; $P = 0.009$, respectively). Natural mortality, without any specific symptoms, remained at very low rates (⩽2.1%).

3.1.3. Experiment 3

The emergence of whitefly adults in bioassays of parasitization by *E. formosa* of third-instar larvae of *T. vaporariorum* contaminated with entomopathogenic fungal inocula in the first 2–4 days following parasitization depended on treatments ($F = 40.72$; $df = 5, 18$; $P < 0.001$) (Table 3). Similar to the previous series of assays on third-instar larvae of *T. vaporariorum*, emergence rates were statistically high in controls (95.9%), in *L. muscarium*-contaminated larvae (77.2%), and in *B. Bassiana*-contaminated larvae (91.8%), and low in larvae exposed to *E. formosa* (5.8%) alone or in combination with *L. muscarium* (4.6%) or *B. Bassiana* (4.0%) (Table 3). Mortality due to fungal formulations did not exceed 22.8% ($F = 0.44$; $df = 3, 12$; $P = 0.729$). Both parasitization and parasitoid emergence rates reached 60–67% without interference between *E. formosa* and the entomopathogenic fungi ($F = 0.018$; $df = 2, 9$; $P = 0.982$ and $F = 0.115$; $df = 2, 9$; $P = 0.893$, respectively). Natural mortality was lower than 6.4%, with the exception of that for whitefly larvae exposed solely to *E. formosa* (27.1%) ($F = 441.12$; $df = 5, 18$; $P < 0.001$).

3.1.4. Greenhouse trials 1

Artificial infestation produced relatively homogeneous *T. vaporariorum* populations, ranging from 40.5 to 57.4 larvae per sampled leaf ($F = 0.074$; $df = 3, 52$; $P = 0.975$) and without any significant difference between control and treatment ($F = 0.505$; $df = 3, 27$; $P = 0.617$) (Table 4). At day 19 post-treatment, fungus-induced mortality reached 36.6%, 39.0%, and 66.1% in plants treated with *L.muscarium, I. fumosorosea,* and *B. bassiana*, respectively, whereas natural mortality in controls was 14.7%. ($F = 11.16$; $df = 3, 52$; $P < 0.0001$) (Table 4). Despite the significant effects of the three fungus-based formulations, it was only on plants treated with the *B. bassiana*-based formulation that the proportion of living larvae was significantly lower than on the control plants (25.6% versus 51.5%) ($F = 4.94$; $df = 3, 52$; $P = 0.0043$). Mortality in the controls consisted mainly of parasitization by *E. formosa* (33.7%).

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Table 4
Interaction between entomopathogenic fungi, *Lecanicillium muscarium, Beauveria bassiana, Isaria fumosorosea,* and natural enemies of *Trialeurodes vaporariorum* in greenhouse tomato crop. Data recorded at day 19: number of whitefly larvae per sampled leaf, emergence rate and mortality due to predation, parasitization, and to fungus infection or to other causes.

	No. whiteflies/sampled leaf live & dead[a]	Emerged Whitefly adults[b]	Whitefly larval mortality[c]		
			Parasitization (black pupae)	Predation	Natural and fungus-induced mortality
Control	1.57 ± 0.13[a] (57.4)	45.10 ± 3.35[a] (51.5%)	33.80 ± 3.88[a] (33.7%)	(0%)	20.97 ± 2.80[c] (14.7%)
L. muscarium as Mycotal	1.53 ± 0.10[a] (45.3)	46.97 ± 4.21[a] (53.8%)	14.69 ± 3.43[b] (9.5%)	(0%)	36.14 ± 3.62[b] (36.6%)
B. bassiana as Naturalis	1.51 ± 0.14[a] (53.6)	28.21 ± 4.43[b] (25.6%)	11.76 ± 3.84[b] (7.9%)	(0%)	54.49 ± 6.20[a] (66.1%)
I. fumosorosea as PreFeRal	1.49 ± 0.10[a] (40.5)	45.33 ± 3.53[a] (50.1%)	15.12 ± 3.32[b] (10.0%)	(0%)	37.54 ± 3.44[b] (39.0%)

[a,b,c] Means within a column followed by the same letter are not significantly different (ANOVA procedure; $\alpha = 0.05$; SNK test).
[a] Mean number of whitefly larvae recorded on each leaf ($x \pm$ sem) expressed as logarithmic value [log ($x + 1$)]. Corresponding number in brackets.
[b] Mean survival ($x \pm$ sem) expressed as angular value (arcsin $\sqrt{}$ [(number of alive larvae +3/8)/total number of whiteflies +3/4)]. Emergence rates (%) in brackets.
[c] Mean larval mortality ($x \pm$ sem) expressed as angular value (arcsin $\sqrt{}$ [(number of parasitized larvae +3/8)/total number of whiteflies +3/4)]. Mortality rates (%) in brackets.

L. muscarium, B. bassiana and *I. fumosorosea* killed young whitefly larvae and limited parasitization to 10% or less.

3.2. Fungus-*M. caliginosus* interactions

3.2.1. Experiment 4

The survival rate of second-instar larvae of *M. caliginosus* in response to *L. muscarium* and *B. bassiana* formulations applied by spraying directly on larvae and by 72-h exposure to fungus-treated leaves was not significantly affected ($F = 0.180$; $df = 2, 9$; $P = 0.838$ and $F = 1.46$; $df = 2, 9$; $P = 0.283$, respectively) (Table 5). In the first type of contamination, survival rates ranged from 81.9% to 86.2% and in the second, from 83.8% to 97.1%.

3.2.2. Experiment 5

A similar absence of detrimental effects of both *B. bassiana*- and *L. muscarium*-based formulations was observed when second-instar larvae of *M. caliginosus* were exposed for 72 h to *T. vaporariorum* prey contaminated 24 h, 72 h, and 5 d prior to feeding ($F = 0.243$; $df = 2, 9$; $P = 0.789$, $F = 0.136$; $df = 2, 9$; $P = 0.874$, and $F = 0.085$; $df = 2, 9$; $P = 0.919$, respectively) (Table 6). Survival rates ranged from 81.9 to 86.2% in larvae directly fungus-sprayed and from 83.8 to 97.1% in larvae exposed to fungus-treated plants.

3.2.3. Greenhouse trials 2

Infestation of tomato plants by *M. caliginosus* adults and larvae was relatively homogenous in both experimental greenhouses,

Table 5
Susceptibility of second-instar *Macrolophus caliginosus* to *Lecanicillium muscarium* and *Beauveria bassiana* formulations applied by spraying directly on larvae or by contamination of the leaves as a substratum for larvae.

	Survival of *M. caliginosus* larvae contaminated directly[a]	Survival of *M. caliginosus* larvae exposed to contaminated foliage[a]
Control	68.2 ± 8.6 (86.2%)	70.1 ± 7.2 (88.4%)
L. muscarium	64.8 ± 11.3 (81.9%)	66.3 ± 14.4 (83.8%)
B. bassiana	67.2 ± 1.0 (85.0%)	80.1 ± 12.7 (97.1%)

[a] Means of data ($x \pm$ sem), expressed as angular value [arcsin $\sqrt{}$ (number of emerged adults/initial number of *M. caliginosus* larvae)]. Survival rates (%) in brackets.

Table 6
Susceptibility of second-instar *Macrolophus caliginosus* exposed for 72 h to *Trialeurodes vaporariorum* prey fungus-inoculated 24 h, 72 h, and 5 days before.

	Survival of *M. caliginosus* larvae[a]		
	A: Prey contaminated 24 h prior predation	B: Prey contaminated 72 h prior predation	C: Prey contaminated 5 d prior predation
Control	72.0 ± 12.2 (90.4%)	68.9 ± 6.7 (87.0%)	69.8 ± 5.9 (88.1%)
L. muscarium	73.7 ± 2.8 (93.9%)	66.4 ± 3.9 (84.0%)	72.0 ± 12.2 (90.4%)
B. bassiana	73.2 ± 4.8 (91.7%)	67.2 ± 8.9 (85.0%)	69.8 ± 5.9 (88.1%)

[a] Means of data (x ± SEM) expressed as angular value [arcsin $\sqrt{}$ (number of emerged adults/initial number of *M. caliginosus* larvae)]. Survival rates (%) in brackets.

Table 7
Effect of two successive applications of *Lecanicillium muscarium*-based products (on April 18 and 26, just after sampling) on *Macrolophus caliginosus* populations to control *Trialeurodes vaporariorum* larvae in tomato crop in climatic regulated greenhouses. Counts of bugs per sampled tomato plant.

	No. *M.caliginosus* per tomato plant per sampling date[a]					
	Treatments	11/04[b]	18/04[c]	26/04[d]	02/05[e]	09/05[f]
Greenhouse 1	Control	4.3 ± 0.5	6.3 ± 1.2	5.6 ± 0.7	4.3 ± 0.4	4.0 ± 0.7
	L. muscarium as Mycotal powder	4.4 ± 0.9	7.1 ± 0.8	6.7 ± 0.9	4.0 ± 0.7	4.0 ± 0.9
	L. muscarium as Mycotal oil formulation	4.3 ± 0.7	6.3 ± 1.0	5.8 ± 0.9	4.7 ± 0.7	4.6 ± 0.4
Greenhouse 2	Control	5.3 ± 0.5	7.6 ± 0.7	6.3 ± 0.8	5.8 ± 1.0	7.8 ± 1.2
	L. muscarium as Mycotal powder	5.3 ± 0.8	8.0 ± 1.0	7.3 ± 0.9	5.8 ± 0.8	6.7 ± 1.8
	L. muscarium as Mycotal oil formulation	4.7 ± 0.8	9.0 ± 0.9	7.8 ± 1.1	4.0 ± 0.5	5.8 ± 1.4

[a] Regular distribution of insects (sampling unit: tomato plant).
[b,c,d,e,f] One way ANOVA of counts for each sampling ($Fb = 0.398$; $df = 5.66$; $P = 0.848$; $Fc = 1.185$; $df = 5.66$; $P = 0.326$; $Fd = 0.873$; $df = 5.66$; $P = 0.504$; $Fe = 1.271$; $df = 5.66$; $P = 0.287$; $Ff = 1.701$; $df = 5.66$; $P = 0.147$).

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with the number of bugs per plant ranging from 3.4 to 5.0 ($F = 0.502$; $df = 5, 66$; $P = 0.773$) on April 11, and from 5.5 to 8.6 ($F = 1.61$; $df = 5, 66$; $P = 0.169$) on April 18 (Table 7). On April 26 (i.e., 8 days after the first treatment with the *L. muscarium*-based formulation), bug infestations reached 5.6–7.8 individuals per plant without any difference between fungus-treated plants and controls ($F = 0.870$; $df = 5, 66$; $P = 0.506$). On May 2 and 9, infestation levels varied from 3.5 to 6.6, without any detrimental effect of the *L. muscarium* treatments on the *M. caliginosus* population ($F = 0.968$; $df = 5, 66$; $P = 0.444$, and $F = 1.671$; $df = 5, 66$; $P = 0.154$).

4. Discussion

The results from both bioassays and greenhouse trials suggest that none of the combinations of the parasitoid, *E. formosa*, and any of the three tested entomopathogenic fungi affected the total mortality of larval populations of *T. vaporariorum*. The mortality of *T. vaporariorum* larvae treated in the second instar revealed the capacity of both *B. bassiana*- and *L. muscarium*-based formulations and of *E. formosa* to kill the host separately or in association. Because of its higher pathogenic activity (under our test conditions), *L. muscarium* provoked a large proportion of mycoses in larvae exposed to parasitization. In contrast, in larvae treated with *B. bassiana* and exposed to *E. formosa*, the efficacy of parasitization was higher because of a lower pathogenic activity of the fungus. Bioassays carried out on third-instar larvae of *T. vaporariorum* showed a low susceptibility of these older larvae to both tested fungi. Consequently, mortality recorded in larvae subjected to combined treatments by consecutive exposures or at 2–4 days post-parasitization was mainly due to the development of the parasitoid.

Greenhouse trials carried out by Ramakers and Samson (1984) showed that, despite a decrease in the parasitization rate, a combined application of *E. formosa* and *Aschersonia aleyrodis* Webber provided better control of whitefly larvae than that of the parasitoid or of the fungus alone. Fransen and van Lenteren (1994) reported a significant reduction in *E. formosa* parasitization by *A. aleyrodis* when *T. vaporariorum* larvae were treated with the fungus in the first 3 days following parasitization. In contrast, treatment with *A. aleyrodis* 4, 7 or 10 days after parasitization did not significantly affect parasitization rates. According to these authors, the rather sudden change from low to high survival of parasitized hosts when treated with *A. aleyrodis* 4 days after parasitization, despite the high number of infected unparasitized larvae, coincided with the hatching of the parasitoid larva from the egg inside the host. Interestingly, they noted that parasitoids emerging from treated hosts showed no differences in reproduction compared with parasitoids emerging from untreated hosts and concluded that both natural enemies of the whitefly are largely compatible. Field experiments on confined populations of *Aphelinus asychis* Walker (Hymenoptera: Aphelinidae) revealed an additive effect of *I. fumosorosea* and *A. asychis* with regard to aphid control, without any detrimental effects on the parasitism rates or parasitoid emergence when the two biocontrol agents were used together (Mesquita et al., 1997; Mesquita and Lacey, 2001). More recently, Rashki et al. (2009) studied the effects of *B. bassiana* on the biological characteristics and life cycle of *Aphidius matricariae* Haliday (Hymenoptera; Braconidae) in relation to the host, *Myzus persicae* Sulzer (Homoptera: Aphididae). The authors concluded that, despite a lower intrinsic rate of increase in parasitoids developed in fungus-infected hosts compared with those that developed in uninfected hosts, their net productive rate was not affected. Most host-parasitoid–pathogen interactions appeared to be detrimental to the parasitoid, mainly because of the premature death of the host due to infection by the fungus (Burleigh, 1975; Los and Allen, 1983; Goh et al., 1989). The time interval between exposure to the parasitoid and application of the fungus was often determinant. Infection of early instar parasitoid larva in freshly parasitized hosts seems to provide a competitive advantage to the fungus, whereas older parasitoid larvae are able to complete their development (Powell et al., 1986; Askary and Brodeur, 1999; Mesquita and Lacey, 2001; Rashki et al., 2009).

Second-instar larvae of *M. caliginosus* were not susceptible to *L. muscarium* or *B. bassiana* formulations after any mode of contamination, by spraying directly on larvae or on the foliar feeding substrate or by contaminated *T. vaporariorum* prey. Greenhouse trials showed that *M. caliginosus* populations treated with fungi were not significantly affected compared to controls. Fargues et al. (2005) observed high levels of natural infestation by *M. caliginosus* in both conventional and "opened" greenhouses during an experimental series studying the effect of the climatic management of Mediterranean greenhouses on the control of *T. vaporariorum* by *L. muscarium*-based formulations. Mortality in controls resulted mainly from predation, with 51% of whitefly larvae predated in the conventional tunnel and 55% in the highly ventilated tunnel. In fungus-treated whitefly populations, mortality by predation did not exceed 13%, and fungus-induced mortality reached 93% and 81%, respectively. The authors noted that fungus-induced mortality occurred mainly in young whitefly larvae, whereas predation started later, when larvae surviving the fungus application reached the fourth larval instar. During these trials, examination of samples never revealed signs of fungal infection in *M. caliginosus* populations (~~Fargues et al., unpublished data~~). Obviously, the host-predator-pathogen interaction revealed competition among the biocontrol agents when the host populations are susceptible to both of them. The control of first- and second-instar larvae of *T. vaporariorum* and *Bemisia tabaci* Gennadius (Homoptera: Aleyrodidae) may provide this type of competitive condition because of their high susceptibility to entomopathogenic fungi (Fransen et al., 1987; Osborne et al., 1990; Vidal et al., 1997, 1998, 2003; Landa et al., 1994; Fargues et al., 2003). In contrast, a combination of biocontrol agents could be favorable for controlling heterogeneous host populations when there is high difference in susceptibility according to the larval stage of development.

5. Uncited reference

Fargues (2003). Q4

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