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Long-Term Field Efficacy of Entomopathogenic Fungi Against *Tetranychus urticae*: Host Plant- and Stage-Specific Responses

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Abstract

The two-spotted spider mite, *Tetranychus urticae* Koch, is a major agricultural pest whose control is increasingly constrained by resistance to synthetic acaricides. This study evaluated the long-term field efficacy of three commercial entomopathogenic fungal (EPF) biopesticides—Velifer® (*Beauveria bassiana*), Metab® (*B. bassiana* + *Metarhizium anisopliae*), and Botanigard® (*B. bassiana*)—against larval and protonymph stages of *T. urticae* on two host plants, Italian cypress (*Cupressus sempervirens*) and sweet orange (*Citrus sinensis*). Two foliar applications were conducted during the 2023 growing season (25 May and 25 July), and mite populations were monitored for 140 days after the final application. A randomized complete block design was used, and efficacy was calculated using the Henderson–Tilton formula. All EPF treatments significantly reduced mite populations compared with the untreated control throughout the monitoring period. Velifer consistently achieved the highest suppression of larval populations, particularly on *C. sinensis*, with efficacy comparable to the chemical standard. Botanigard showed more gradual but sustained population reduction over time, whereas Metab exhibited lower but stable efficacy in all cases. Treatment performance was strongly influenced by host plant species and mite developmental stage, with larvae consistently more susceptible than protonymphs. On *C. sinensis*, Velifer achieved the highest larval suppression (84.6%), comparable to the chemical standard abamectin, while Botanigard and Velifer were most effective on *C. sempervirens*. Survival analysis confirmed isolate- and host-dependent differences in hazard effects over time. These results demonstrate that EPF-based products can provide sustained, long-term suppression of *T. urticae* under field conditions, supporting their integration into integrated pest management programs.

Keywords: *Beauveria bassiana*; *Metarhizium anisopliae*; biological control; two-spotted spider mite; host plant effect; integrated pest management

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1. Introduction

Tetranychus urticae Koch (Acari: Tetranychidae), known as the two-spotted spider mite, is one of the most destructive and economically important arthropod pests worldwide. It is highly polyphagous, infesting more than 1200 plant species across agricultural, horticultural, and ornamental systems, including fruit trees, vegetables, field crops, and

forest nursery plants [1–11]. Its pest status is driven by a combination of biological traits, including a short generation time, high fecundity, arrhenotokous parthenogenesis, rapid population growth, and strong adaptive capacity to diverse host plants and environmental conditions [12–15]. Feeding by both immature and adult stages causes extensive damage to mesophyll tissues, disrupts photosynthesis, and induces chlorosis, bronzing, necrosis, and premature leaf senescence, often accompanied by webbing, resulting in yield losses and reduced crop quality [16–22].

Control of *T. urticae* has historically depended on synthetic acaricides. However, increasing evidence demonstrates that the long-term effectiveness of these compounds is severely compromised by the rapid development of resistance to multiple active ingredients [23–29]. This resistance evolution is driven by the mite's high reproductive potential and short life cycle, while repeated chemical applications additionally disrupt natural enemy communities and pose risks to human health and the environment [15,30–35]. As a result, there is growing interest in alternative and complementary control strategies that are both effective and compatible with integrated pest management (IPM) principles.

Among non-chemical approaches, entomopathogenic fungi (EPFs) have attracted increasing attention as biological control agents against spider mites. EPFs infect their hosts through direct contact, adhering to the cuticle, penetrating via enzymatic and mechanical processes, and proliferating internally within the hemocoel, causing host death [36–38]. This mode of action differs fundamentally from that of conventional acaricides, reducing the likelihood of cross-resistance and making EPFs suitable for resistance management strategies [23,36]. Although some EPF species such as *B. bassiana* and *M. anisopliae* exhibit a broad host range, pathogenicity is strain-specific, and commercially registered formulations are regarded as selective and environmentally safe when applied according to label recommendations [37–39].

Numerous laboratory and short-term semi-field studies have demonstrated the potential of EPFs to suppress *T. urticae* populations [19,39,40]. These studies consistently report higher susceptibility of early developmental stages, particularly larvae, compared with later instars and adults. However, most existing evaluations are limited to short exposure periods, often ranging from a few days to several weeks, and focus primarily on acute mortality under controlled conditions. Consequently, critical aspects such as long-term persistence, cumulative population suppression, host plant effects, and performance across multiple mite generations remain poorly documented under realistic field conditions.

Host plant species can influence spider mite population dynamics and susceptibility to control measures through differences in leaf morphology, canopy architecture, secondary metabolites, and microclimatic conditions [4,11]. These plant-mediated effects may also affect EPF performance by altering spore deposition, humidity retention, and infection success. Despite their potential importance, host plant effects are rarely incorporated into EPF efficacy studies, particularly in long-term field experiments.

Therefore, there is a clear need for extended field-based evaluations that assess the durability and practical relevance of EPF-based biopesticides against *T. urticae* under commercial conditions. The objectives of the present study were to: (i) evaluate the long-term (140-day) field efficacy of three commercially available EPF-based biopesticides against *T. urticae*; (ii) compare their performance on two contrasting host plant species, *C. sempervirens* and *C. sinensis*; (iii) assess stage-specific susceptibility of larval and protonymph stages; and (iv) benchmark EPF performance against a conventional chemical acaricide. By moving beyond short-term assessments, this study aims to provide biologically and agronomically relevant evidence supporting the integration of EPFs into sustainable IPM programs for spider mite management.

2. Materials and Methods

2.1. Experimental Design

Field trials were conducted during the 2023 growing season in two locations in western Greece. The Cupressus trial was established in a commercial nursery in Lappa Forest Nursery (Achaia, Greece) using five-year-old *C. sempervirens* cv. 'Stricta' grown in 25 L containers. Trees were spaced 2 m apart within rows and 3 m between rows. The Citrus trial was conducted in a commercial orchard in Vonitsa (Aitoloakarnania, Greece) using mature *C. sinensis* (cv. Valencia) trees.

Two foliar applications were performed on 25 May and 25 July 2023, timed according to pest phenology. Applications were made in the late evening (after 19:00) to reduce UV exposure and enhance humidity, conditions favorable for fungal infection. Mite populations were monitored one day before the first application (Day 0) and at 7, 14, 21, 28, 56, 84, 102, and 140 days after the final application.

The experimental design was a randomized complete block design (RCBD) with five replications per treatment. Each tree represented an independent experimental unit, and blocks were arranged to account for minor gradients in light exposure. Treatments were applied using a calibrated motorized backpack mistblower delivering 1500 L ha^{-1} (approximately 1.5 L per tree), ensuring complete canopy coverage. Continuous on-site microclimatic measurements were not available at the commercial nursery. However, average temperature and relative humidity data for the experimental period were obtained from the nearest regional meteorological station to provide contextual environmental information. Mean temperatures during the study period were typical of the region and season, and relative humidity levels were within ranges reported as favorable for entomopathogenic fungal activity.

2.2. Biopesticides

Three commercial entomopathogenic fungal (EPF)-based biopesticides were evaluated in this study: Velifer® (*B. bassiana* strain PPRI 5339) (BASF Hellas, Imittos, Greece), Metab® (*B. bassiana* + *M. anisopliae*) (Sacom Hellas, Athina, Greece), and Botanigard® (*B. bassiana*) (K&N Efthymiadis, Sindos, Greece). All products were applied according to manufacturer recommendations. Velifer® was applied at a rate of 100 mL product per 100 L water (equivalent to 1.0 L ha^{-1}), Metab® at 150 mL per 100 L water (1.5 L ha^{-1}), and Botanigard® at 100 mL per 100 L water (1.0 L ha^{-1}).

The chemical acaricide abamectin (Valmec® 1.8 EC) (Paterna, Spain) was applied at 40 mL per 100 L water (0.6 L ha^{-1}) and served as a positive control, while water-treated plants were used as the untreated control. All treatments were applied using a calibrated backpack mistblower delivering approximately 1500 L ha^{-1} (≈1.5 L per tree), ensuring uniform canopy coverage. All products were applied at manufacturer-recommended field rates.

2.3. Field Control Experiments with Mites and Fungus

Naturally occurring *T. urticae* populations were present at both sites and were supplemented uniformly by attaching infested bean leaves (*Phaseolus vulgaris*) to the canopy two weeks before the first application. Mite densities were monitored weekly until populations exceeded 10 mobile mites per sampling unit. At each sampling date, leaves (for citrus) or 10 cm branch tips (for cypress) were collected and examined under stereoscopic and compound microscopes. Only larval and protonymph stages were counted; eggs, deutonymphs, and adults were excluded to ensure consistent stage-specific comparisons. In the laboratory, the identity of *T. urticae* was verified using both stereoscopic and compound microscopy. General morphology was examined with a stereoscope (SZ51, Olympus (Olym-

pus Corporation, Tokyo, Japan)) at 40× magnification, while detailed diagnostic features were observed using a compound microscope (Primo Star, Carl Zeiss Microscopy GmbH, Jena, Germany) at 100–400× magnification. All bioassay observations were conducted under controlled laboratory conditions (25 ± 2 °C, 65 ± 5% RH, 16:8 h L:D photoperiod). Prior to treatment, 40 leaves from 15 trees were selected randomly from each plot (tree). Sampled leaves were placed in paper bags and transported to the Laboratory of Productive Agriculture and Plant Health, University of Ioannina, Department of Agriculture (Arta), 40 leaves from each tree; we took them to the laboratory and then selected 1 leaf from each direction from the middle and upper part of the canopy (a total of 8 leaves) and counted the mites.to estimate the initial mite population, measuring only larvae and protonymphs (mite eggs, deuteronymphs and adults were not counted). On day 0, larvae and protonymphs were counted randomly, and the treatment was then applied. In total, 9 samplings were performed until 140 DAT.

2.4. Data Analysis

Mite density data were square root transformed prior to analysis. Two-way ANOVA was performed with treatment and sampling date as factors. Mean comparisons were conducted using Bonferroni tests ($\alpha = 0.05$). Treatment efficacy was calculated using the Henderson–Tilton formula. Treatment efficacy values shown in Figure 1 were calculated using the Henderson–Tilton formula, which corrects for population changes in the untreated control. Although Figure 1 visually benchmarks treatments against positive control, the untreated control is inherently incorporated into all efficacy calculations. Survival and hazard effects were analyzed using Cox proportional hazards regression to evaluate temporal and treatment-specific mortality risk. The Cox Regression method [41] was selected to determine the hazard effect of the EPF-biopesticides over *T. urticae*. It is a survival analysis regression model that describes the relation between the event incidence, as expressed by the hazard function, and a set of covariates. Comparison of survival distributions was obtained using Breslow (Generalized Wilcoxon).

Survival data are described and modeled in terms of two related probabilities, survival and hazard. The survival probability (which is also called "the survivor function"), S(t), is the probability that an individual survives from the time origin (e.g., beginning of treatment) to a specified future time, t.

The hazard probability is usually denoted by h(t) or λ(t) and refers to the probability that an individual who is under observation at time t, has an event at that time. It represents the instantaneous event rate for an individual who has already survived by time t. Thus, while the survivor function reflects the cumulative non-occurrence of an event, the hazard function focuses on the occurrence of an event.

The mathematical equation of the Cox model is:

$$h(t) = h0(t) \times \exp\{b1 \times 1 + b2 \times 2 + \cdots + bpxp\}$$

where the hazard function h(t) is dependent on (or determined by) a set of p covariates (x1, x2, . . ., xp), whose impact is measured by the size of the respective coefficients (b1, b2, . . ., bp). The term h0 is called the baseline hazard and is the value of the hazard if all the xi are equal to zero (the quantity exp(0) equals 1). The 't' in h(t) reminds us that the hazard may (and probably will) vary over time.

Efficacy Calculation: The percent reduction in mite population for each treatment was calculated using the Henderson–Tilton formula:

$$\text{Efficacy (\%)} = [1 - (Ta * Cb)/(Tb * Ca)] * 100$$

where Ta = mites in treatment after application; Tb = mites in treatment before application; Ca = mites in control after application; Cb = mites in control before application.

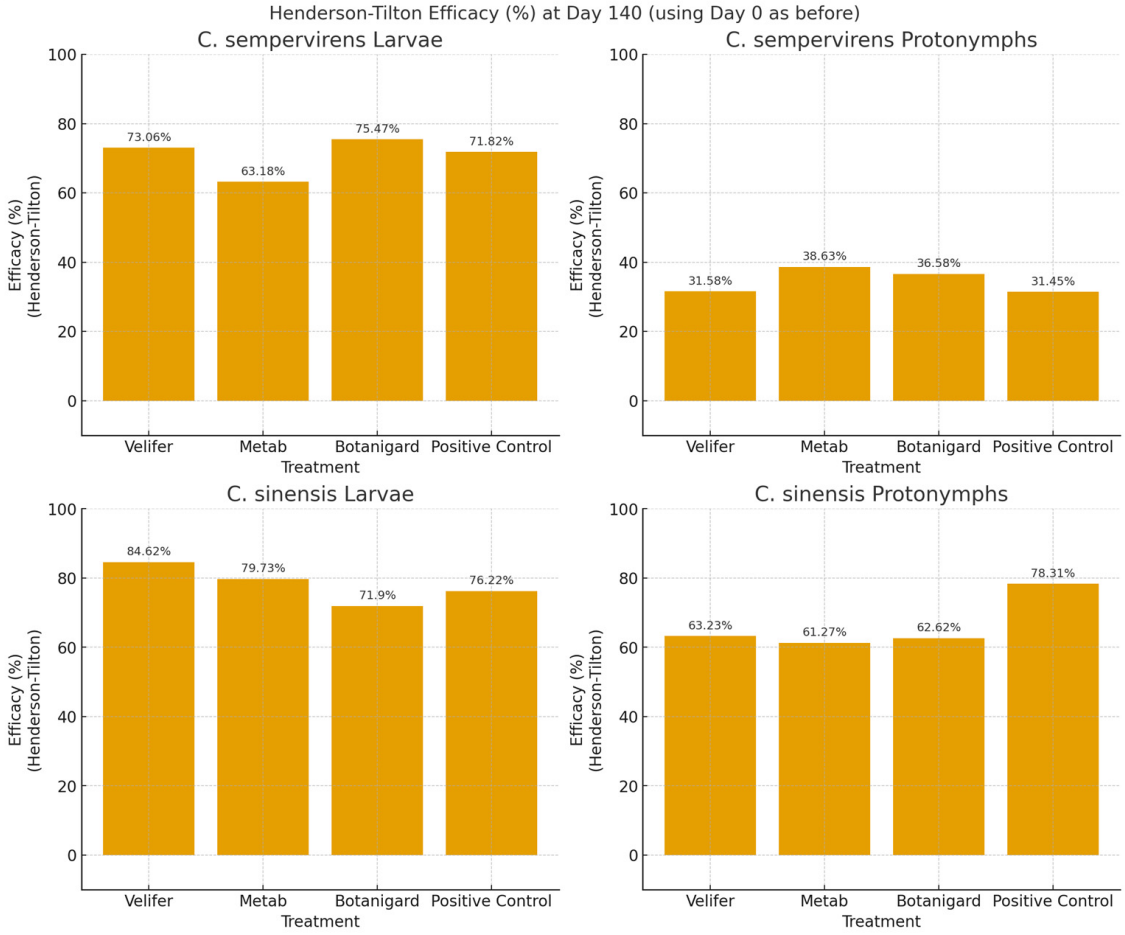


Figure 1. Corrected efficacy (%) of entomopathogenic fungal biopesticides against *T. urticae*, calculated using the Henderson–Tilton formula. Efficacy values account for population changes in the untreated control, while treatments are visually benchmarked against the positive control for comparative purposes. Efficacy values are presented descriptively to illustrate long-term treatment performance; statistical significance among treatments was evaluated using ANOVA on population densities and Cox regression survival analysis.

3. Results

All EPF treatments significantly reduced *T. urticae* populations compared with the untreated control across both host plants and throughout the 140-day monitoring period. Larval stages were consistently more susceptible than protonymphs. Treatment efficacy varied with host plant species, with higher suppression observed on *C. sinensis* than on *C. sempervirens*. Velifer and Botanica frequently achieved population suppression comparable to abamectin, particularly against larvae (Figure 1). On *C. sempervirens*, Botanigard and Velifer achieved levels of larval control comparable to the chemical control, whereas Metab showed moderate but stable suppression. On *C. sinensis*, Velifer provided the highest larval control, while efficacy against protonymphs remained lower and more variable across treatments (Figure 2). These results, supported by significant ANOVA effects for collection day, treatment and their interaction ($p < 0.001$), indicate that efficacy varied by both species and life stage; larvae were generally better controlled than protonymphs, larvae on *C. sinensis* were more responsive to treatments than *C. sempervirens*, and the magnitude

of control depended on initial population levels (Tb) and control growth (Cb → Ca) as accounted for by the Henderson–Tilton correction.

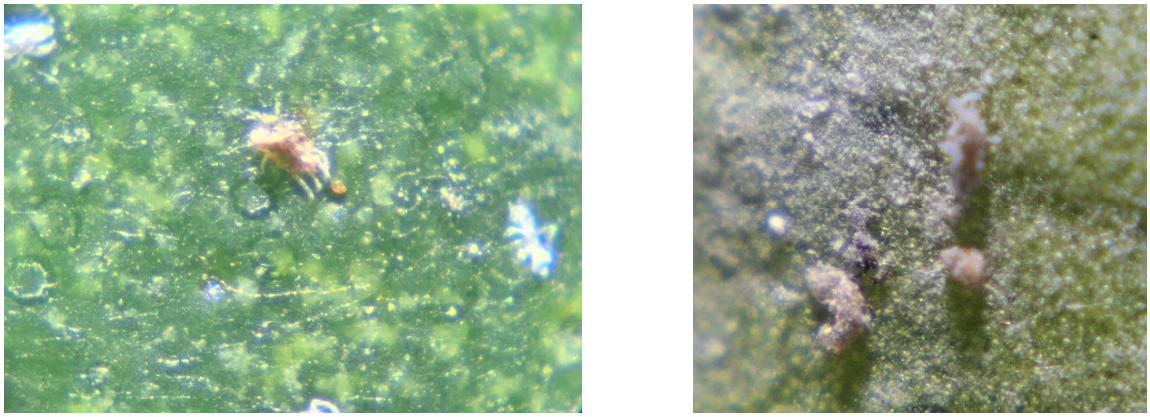


Figure 2. Visual documentation (magnification ×40) of *T. urticae* protonymphs and treatment effects in the field trials (**left**) A live *T. urticae* protonymph on a leaf of *C. sempervirens* representing the target pest stage prior to biopesticide application. (**right**) Dead *T. urticae* protonymphs on a leaf of *C. sinensis* after Velifer treatment.

Application of the three entomopathogenic fungal formulations—Velifer, Metab, and Botanigard—significantly suppressed *T. urticae* populations on both *C. sempervirens* and *C. sinensis* across the 140-day monitoring period. In all cases, treated plots exhibited lower larval and protonymph densities than the untreated negative control, which showed continuous population growth throughout the experiment. On *C. sempervirens*, larval densities increased during the early post-treatment intervals (days 7–28) for all treatments, followed by a gradual decline or stabilization (Tables 1 and 2). By day 140, larval populations in Velifer, Metab, and Botanigard treatments were reduced to 24.2 ± 2.8, 34.1 ± 2.5, and 25.9 ± 1.2 individuals per sample unit, respectively, compared with 202.8 ± 30.2 in the negative control. A similar pattern was observed for protonymphs, with final densities of 44.1 ± 1.9 (Velifer), 44.9 ± 2.1 (Metab), and 45.3 ± 2.8 (Botanigard), in contrast to 198.6 ± 32.8 in the negative control. Positive control also maintained comparatively low densities for both stages, indicating consistent suppression across treated groups. Population trends on *C. sinensis* closely mirrored those observed on *C. sempervirens*. Larval densities under Velifer, Metab, and Botanigard peaked at early intervals and subsequently declined, resulting in final measurements of 16.6 ± 2.1, 24.6 ± 2.9, and 29.3 ± 2.8 at day 140, respectively (Tables 3 and 4). These values remained lower than the negative control, which continued to increase and reached 217.1 ± 35.1. Protonymph populations displayed the same overall pattern, ending at 34.7 ± 1.5 (Velifer), 44.2 ± 2.1 (Metab), and 35.0 ± 2.3 (Botanigard), whereas the negative control rose to 243.6 ± 29.4 by the final sampling date. The results indicated significant effects of treatment, population collection days, and their interaction on both larvae and protonymphs of *C. sempervirens* and *C. sinensis* ($p < 0.001$) (Figure 1, Table 5).

The efficacy of treatments was significantly influenced by the host plant species, the biological stage of the pest, and the duration of exposure. Time-series analysis revealed sustained suppression rather than rapid knockdown, with population divergence between treated and untreated plots becoming more pronounced over time. Cox regression analysis indicated isolate-specific and host-dependent hazard effects, with some treatments exhibiting increasing cumulative mortality over extended exposure periods (Table 6). The effects of Velifer, Metab, and Botanigard on the survival of nymph and protonymph stages of *C. sempervirens* and *C. sinensis* were quantified using survival proportions and hazard effects as indicators of mortal-

ity risk across 28, 84, and 140 days of exposure. On *C. sempervirens*, the Metab demonstrated lower performance against both nymphs and protonymphs, maintaining lower mortality (final survival effect: 0.733 and 0.833, respectively) and often outperformed the other two treatments. In contrast, the virulence of Velifer was highly variable, showing high efficacy against nymphs but not strong suppression of protonymphs at 140 days (survival effect: 0.967). A similar plant-mediated effect was observed on *C. sinensis*, where all treatments, including the positive control, exhibited reduced overall efficacy compared to their performance on *C. sempervirens*. Across both plants, the protonymph stage was more difficult to suppress with the biological treatments than the nymph stage. Temporal dynamics revealed that while Velifer exerted a strong and early effect, especially for the nymphs of *T. urticae*, the hazard effect of Botanigard increased over time, indicating a slower, more cumulative mode of action. Furthermore, the host plant species significantly modulated the hazard, with all treatments presenting a higher effective hazard profile on *C. sinensis* compared to *C. sempervirens*. The positive control typically generated a moderate but stable hazard over time, which was frequently surpassed by Velifer and the later-stage efficacy of Botanigard.

Table 1. Mean (±Sd) population density of larvae of *T. urticae* on *C. sempervirens* at different intervals following foliar application of three entomopathogenic fungal treatments (Velifer, Metab, and Botanigard), along with positive and negative controls. All treatments were applied once at the beginning of the experiment, and populations were monitored for 140 days under field conditions. Different exposure intervals represent post-treatment collection days. Means represent values per sample unit. Higher values indicate population growth, while lower values reflect fungal suppression of mite development and reproduction. Means of the same column followed by the same letter do not differ significantly. Only selected sampling dates are shown for clarity; full temporal data are provided in Appendix A Table A1.

Treatment	Dose (mL/100 L)	Larval Population Density (Mites/Sampling Unit) at Selected Days After Treatment (DAT)			
		0 DAT	28 DAT	56 DAT	140 DAT
Velifer	100	>10	46.6 ± 2.7 a	20.0 ± 7.7 a	24.2 ± 2.8 a
Metab	100		53.3 ± 1.8 a	78.3 ± 4.3 b	34.1 ± 2.5 a
Botanigard	100		53.3 ± 2.1 a	22.8 ± 2.5 a	25.9 ± 1.2 a
Valmec (Positive Control)	50		47.6 ± 3.4 a	39.3 ± 1.5 c	21.4 ± 2.9 a
dd H_2O (Negative Control)	-		186.3 ± 9.5 b	156.6 ± 12.4 d	202.8 ± 32.2 b

Table 2. Mean (±Sd) population density of protonymphs of *T. urticae* on *C. sempervirens* at different intervals following foliar application of three entomopathogenic fungal treatments (Velifer, Metab, and Botanigard), along with positive and negative controls. All treatments were applied once at the beginning of the experiment, and populations were monitored for 140 days under field conditions. Different exposure intervals represent post-treatment collection days. Means represent values per sample unit. Higher values indicate population growth, while lower values reflect fungal suppression of mite development and reproduction. Means of the same column followed by the same letter do not differ significantly. Only selected sampling dates are shown for clarity; full temporal data are provided in Appendix A Table A2.

Treatment	Dose (mL/100 L)	Protonymphs Population Density (Mites/Sampling Unit) at Selected Days After Treatment (DAT)			
		0 DAT	28 DAT	56 DAT	140 DAT
Velifer	100	>10	36.6 ± 2.4 a	47.3 ± 2.2 a	44.1 ± 1.9 a
Metab	100		43.3 ± 3.7 b	63.3 ± 3.9 b	44.9 ± 2.1 a
Botanigard	100		48.6 ± 2.2 b	52.6 ± 1.1 a	45.3 ± 2.8 a
Valmec (Positive Control)	50		37.3 ± 2.4 a	49.2 ± 2.1 a	41.4 ± 3.1 a
dd H_2O (Negative Control)	-		86.6 ± 4.5 c	136.6 ± 23.9 c	198.6 ± 32.8 b

Table 3. Mean (±SE) population density of larvae of *T. urticae* on *C. sinensis* at different intervals following foliar application of three entomopathogenic fungal treatments (Velifer, Metab, and Botanigard), along with positive and negative controls. All treatments were applied once at the beginning of the experiment, and populations were monitored for 140 days under field conditions. Different exposure intervals represent post-treatment collection days. Means represent values per sample unit. Higher values indicate population growth, while lower values reflect fungal suppression of mite development and reproduction. Means of the same column followed by the same letter do not differ significantly. Only selected sampling dates are shown for clarity; full temporal data are provided in Appendix A Table A3.

Treatment	Dose (mL/100 L)	Larval Population Density (Mites/Sampling Unit) at Selected Days After Treatment (DAT)			
		0 DAT	28 DAT	56 DAT	140 DAT
Velifer	100	>10	32.6 ± 2.4 a	13.2 ± 2.8 a	16.6 ± 2.1 a
Metab	100		56.1 ± 1.2 b	34.7 ± 3.2 b	24.6 ± 2.9 a
Botanigard	100		43.3 ± 3.3 a	13.9 ± 2.1 a	29.3 ± 2.8 a
Valmec (Positive Control)	50		34.5 ± 2.8 a	11.8 ± 4.8 a	21.6 ± 1.8 a
dd H_2O (Negative Control)	-		177.2 ± 21.1 c	136.5 ± 25.2 c	217.1 ± 35.1 b

Table 4. Mean (±SE) population density of protonymphs of *T. urticae* on *C. sinensis* at different intervals following foliar application of three entomopathogenic fungal treatments (Velifer, Metab, and Botanigard), along with positive and negative controls. All treatments were applied once at the beginning of the experiment, and populations were monitored for 140 days under field conditions. Different exposure intervals represent post-treatment collection days. Means represent values per sample unit. Higher values indicate population growth, while lower values reflect fungal suppression of mite development and reproduction. Means of the same column followed by the same letter do not differ significantly. Only selected sampling dates are shown for clarity; full temporal data are provided in Appendix A Table A4.

Treatment	Dose (mL/100 L)	Protonymphs Population Density (Mites/Sampling Unit) at Selected Days After Treatment (DAT)			
		0 DAT	28 DAT	56 DAT	140 DAT
Velifer	100	>10	56.6 ± 1.2 a	50.0 ± 0.0 a	34.7 ± 1.5 a
Metab	100		63.6 ± 2.1 a	58.4 ± 1.8 a	44.2 ± 2.1 b
Botanigard	100		58.4 ± 1.9 a	52.8 ± 2.1 a	35.4 ± 2.3 a
Valmec (Positive Control)	50		47.2 ± 1.5 b	49.6 ± 1.9 a	21.1 ± 1.9 a
dd H_2O (Negative Control)	-		86.6 ± 7.6 c	106.4 ± 19.8 b	243.6 ± 29.4 c

Table 5. ANOVA parameters for main effects and associated interactions for mortality levels of *T. urticae*.

Source	df	F	Sig.
Time (Days after Treatment)	7.167	10.144	<0.0001
Treatment	5.167	15.323	<0.0001
Time * Treatment	53.167	14.435	<0.0001

Table 6. Survival and Hazard Effect of entomopathogenic fungi on *T. urticae* nymphs and protonymphs (Cox Regression method).

Plant Species	Biological Stage	Treatment	Exposure Time (Days)	Survival	
				Survival Effect *	Hazard Effect **
C. sempervirens	Nymphs	Velifer	28	0.633	0.899
			84	0.533	1.015
			140	0.333	0.401
		Metab	28	0.933	0.069
			84	0.800	0.223
			140	0.733	0.310
		Botanigard	28	0.767	0.266
			84	0.600	0.511
			140	0.467	0.762
		Positive control	28	0.600	0.511
			84	0.533	0.629
			140	0.400	0.916
	Protonymphs	Velifer	28	0.800	0.223
			84	0.733	0.310
			140	0.867	0.134
		Metab	28	0.967	0.034
			84	0.900	0.105
			140	0.733	0.169
		Botanigard	28	0.967	0.034
			84	0.898	0.108
			140	0.900	0.123
		Positive control	28	0.900	0.105
			84	0.700	0.357
			140	0.900	0.105
C. sinensis	Nymphs	Velifer	28	0.500	0.405
			84	0.400	0.916
			140	0.567	0.568
		Metab	28	0.933	0.069
			84	0.900	0.105
			140	0.700	0.357
		Botanigard	28	0.800	0.223
			84	0.633	0.457
			140	0.500	0.693
		Positive Control	28	0.467	0.334
			84	0.400	0.916
			140	0.433	0.582
	Protonymphs	Velifer	28	0.898	0.108
			84	0.800	0.223
			140	0.433	0.657
		Metab	28	0.967	0.034
			84	0.833	0.182
			140	0.600	0.511
		Botanigard	28	0.800	0.223
			84	0.767	0.266
			140	0.400	0.610
		Positive Control	28	0.933	0.069
			84	0.633	0.457
			140	0.400	0.916

* **Survival effect:** the probability that an individual survives from the time origin (e.g., beginning of treatment) to a specified future time; ** **Hazard effect:** the probability that an individual who is under observation at a time t, has an event at that time. Survival and Hazard effect values are model-based relative estimates derived from Cox proportional hazards regression and should not be interpreted as absolute survival probabilities.

4. Discussion

The present field study demonstrated clear, host- and stage-dependent differences in the long-term efficacy of three commercial entomopathogenic fungal formulations against

T. urticae, with larvae consistently more susceptible than protonymphs and treatment performance varying between *C. sempervirens* and *C. sinensis*.

Most published evaluations of entomopathogenic fungi (EPFs) against *T. urticae* are restricted to laboratory bioassays or short-term semi-field trials, typically lasting from a few days to several weeks [19,39,40]. These studies primarily assess acute mortality or short-term efficacy and therefore provide limited insight into persistence, population rebound, or performance across multiple pest generations under realistic agronomic conditions. In contrast, the present study offers a rare long-term field evaluation extending 140 days after the final application, allowing assessment of sustained population dynamics and treatment durability under commercial nursery and orchard conditions. This extended monitoring period represents a substantial advance over conventional short-duration studies and provides biologically meaningful information for integrated pest management (IPM) decision-making.

The results demonstrate that commercial EPF formulations can provide durable population suppression rather than rapid knockdown, a pattern that contrasts with the immediate but often transient effects observed in short-term studies [19,40]. In our field trials, this pattern was evident across all EPF formulations tested, with Velifer showing earlier population suppression, whereas Botanigard exhibited a more gradual but increasing effect over time. EPF-treated plots showed progressive divergence from untreated controls over time, suggesting cumulative infection processes, delayed mortality, and potential secondary cycling of fungal propagules. Such long-term suppression is particularly relevant for managing multivoltine pests such as *T. urticae*, where maintaining populations below economic thresholds is more critical than achieving short-lived reductions [23,32].

Developmental stage strongly influenced treatment outcomes, with larval stages consistently more susceptible than protonymphs across both host plants. This stage-specific pattern was consistently observed across all sampling dates and treatments, with larval populations showing markedly higher suppression levels than protonymphs throughout the 140-day monitoring period. While stage-dependent susceptibility has been frequently reported in laboratory assays [19], the present study provides field-based confirmation across an extended time frame, demonstrating that this pattern persists under fluctuating environmental conditions. The higher susceptibility of larvae is related to their thinner cuticle and reduced behavioral defenses, whereas protonymphs exhibit increased survivorship and tolerance [19,20]. These findings emphasize the importance of synchronizing EPF applications with population peaks of early immature stages to maximize field efficacy. In our field trials, treatments applied when larval stages were predominant resulted in more pronounced and sustained population suppression, highlighting the practical relevance of stage-targeted EPF applications.

Host plant species significantly modulated EPF performance, with higher efficacy observed on *C. sinensis* than on *C. sempervirens*. Differences in leaf morphology, canopy structure, and microclimatic conditions influenced spore deposition, humidity retention, and fungal persistence, as previously suggested for spider mite–plant interactions [4,11]. Broad-leaved citrus trees may create a more favorable microenvironment for fungal infection than the needle-like foliage of cypress. Importantly, host plant effects are rarely addressed in short-term laboratory or semi-field studies, highlighting the added value of multi-host, long-term field evaluations such as the present work.

When benchmarked against the chemical acaricide abamectin, EPF treatments achieved comparable levels of larval suppression by the end of the monitoring period, supporting their practical relevance for IPM programs. Although chemical acaricides remain effective tools, their intensive use has led to widespread resistance in *T. urticae* populations [26–29]. The distinct mode of action of EPFs—based on cuticular penetration and internal proliferation rather than neurotoxicity—reduces the likelihood of cross-resistance and enhances their value as complementary or rotational control options [23,36,37].

A limitation of the present study is the absence of continuous microclimatic measurements, which are known to influence EPF performance [42,43]. Nevertheless, evening applications were deliberately employed to reduce UV exposure and enhance humidity, and untreated control plots exhibited continuous population growth throughout the experiment. These observations indicate that the long-term suppression observed in EPF-treated plots cannot be attributed solely to seasonal population decline. Future studies integrating detailed environmental monitoring and compatibility with predatory mites will further optimize EPF deployment strategies [39].

This study provides one of the few long-term (140-day) field evaluations of commercial entomopathogenic fungal biopesticides against *T. urticae*, addressing a critical gap in a literature dominated by short-term laboratory and semi-field assays [19,39,40]. By simultaneously assessing host plant effects and developmental stage susceptibility under commercial nursery and orchard conditions, the work offers novel insights into the persistence, cumulative action, and practical performance of EPFs across multiple mite generations. The demonstration of sustained population suppression comparable to a chemical acaricide, rather than transient knockdown, strengthens the evidence for EPFs as durable, resistance-mitigating tools within integrated pest management programs [23,26–29]. These findings advance current understanding of EPF field performance and support their broader adoption in sustainable mite management strategies.

Overall, this study moves beyond short-term efficacy testing and demonstrates that EPF-based biopesticides can provide persistent, host-dependent, and stage-specific suppression of *T. urticae* under realistic field conditions, reinforcing their suitability for sustainable IPM programs.

5. Conclusions

This field study demonstrates that commercially available entomopathogenic fungal biopesticides (Velifer, Metab, and Botanigard) can provide effective and sustained suppression of *T. urticae* populations, particularly targeting vulnerable larval stages, and in some cases, achieving efficacy comparable to synthetic acaricides. Treatment performance was strongly influenced by mite developmental stage, host plant species, and fungal strain, highlighting the importance of timing applications, plant architecture, and selecting appropriate EPF formulations. The distinct mode of action of EPFs, coupled with their prolonged activity and environmental safety, makes them a promising component of integrated pest management programs aimed at mitigating acaricide resistance. Commercial entomopathogenic fungal biopesticides provided effective, host-dependent, and stage-specific long-term suppression of *T. urticae* under field conditions. Their performance, comparable in some cases to chemical acaricides, supports their integration into IPM programs aimed at reducing resistance development and environmental impact. Future studies should further evaluate environmental drivers of EPF persistence and compatibility with natural enemies.

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Appendix A

Table A1. Mean (±Sd) population density of larvae of *T. urticae* on *C. sempervirens* at different intervals following foliar application of three entomopathogenic fungal treatments (Velifer, Metab, and Botanigard), along with positive and negative controls. All treatments were applied once at the beginning of the experiment, and populations were monitored for 140 days under field conditions. Different exposure intervals represent post-treatment collection days. Means represent values per sample unit. Higher values indicate population growth, while lower values reflect fungal suppression of mite development and reproduction. Means of the same column followed by the same letter do not differ significantly.

Treatment	Dose (mL/100 L)	Larval Population Density (Mites/Sampling Unit) at Selected Days After Treatment (DAT)								
		0	7	14	21	28	56	84	102	140
Velifer	100		19.4 ± 2.1 a	24.4 ± 2.9 a	38.3 ± 1.1 a	46.6 ± 2.7 a	20.0 ± 7.7 a	29.6 ± 3.6 a	25.6 ± 1.3 a	24.2 ± 2.8 a
Metab	100		20.0 ± 4.3 a	28.8 ± 3.4 a	49.1 ± 2.2 b	53.3 ± 1.8 a	78.3 ± 4.3 b	60.0 ± 2.9 b	24.4 ± 3.5 a	34.1 ± 2.5 a
Botanigard	100	>10	22.8 ± 4.6 a	23.4 ± 3.9 a	45.4 ± 2.9 b	53.3 ± 2.1 a	22.8 ± 2.5 a	24.4 ± 1.6 a	23.1 ± 2.9 a	25.9 ± 1.2 a
Valmec (Positive Control)	50		16.4 ± 5.1 a	20.0 ± 1.8 a	26.6 ± 3.2 c	47.6 ± 3.4 a	39.3 ± 1.5 c	35.4 ± 2.5 c	13.8 ± 1.9 a	21.4 ± 2.9 a
dd H_2O (Negative Control)	-		43.8 ± 2.7 b	117.4 ± 23.6 c	151.2 ± 12.9 d	186.3 ± 9.5 b	156.6 ± 12.4 d	93.9 ± 13.1 d	219.1 ± 20.8 b	202.8 ± 32.2 b

Table A2. Mean (±Sd) population density of protonymphs of *T. urticae* on *C. sempervirens* at different intervals following foliar application of three entomopathogenic fungal treatments (Velifer, Metab, and Botanigard), along with positive and negative controls. All treatments were applied once at the beginning of the experiment, and populations were monitored for 140 days under field conditions. Different exposure intervals represent post-treatment collection days. Means represent values per sample unit. Higher values indicate population growth, while lower values reflect fungal suppression of mite development and reproduction. Means of the same column followed by the same letter do not differ significantly.

Treatment	Dose (mL/100 L)	Protonymphs Population Density (Mites/Sampling Unit) at Selected Days After Treatment (DAT)								
		0	7	14	21	28	56	84	102	140
Velifer	100		11.1 ± 1.2 a	25.2 ± 2.2 a	28.6 ± 2.5 a	36.6 ± 2.4 a	47.3 ± 2.2 a	39.5 ± 2.4 a	45.2 ± 2.3 a	44.1 ± 1.9 a
Metab	100		12.6 ± 2.1 a	32.4 ± 2.1 a	39.1 ± 1.8 b	43.3 ± 3.7 b	63.3 ± 3.9 b	50.0 ± 3.8 b	54.5 ± 4.5 a	44.9 ± 2.1 a
Botanigard	100	>10	12.3 ± 1.8 a	23.3 ± 1.2 a	25.6 ± 2.4 a	48.6 ± 2.2 b	52.6 ± 1.1 a	34.4 ± 2.6 c	43.3 ± 2.8 a	45.3 ± 2.8 a
Valmec (Positive Control)	50		10.4 ± 1.1 a	19.5 ± 1.9 a	21.1 ± 2.8 a	37.3 ± 2.4 a	49.2 ± 2.1 a	25.9 ± 2.1 c	33.3 ± 1.9 b	41.4 ± 3.1 a
dd H_2O (Negative Control)	-		34.2 ± 3.2 b	42.2 ± 2.5 b	51.6 ± 4.8 c	86.6 ± 4.5 c	136.6 ± 23.9 c	144 ± 32.1 d	193.4 ± 35.3 c	198.6 ± 32.8 b

Table A3. Mean (±SE) population density of larvae of *T. urticae* on *C. sinensis* at different intervals following foliar application of three entomopathogenic fungal treatments (Velifer, Metab, and Botanigard), along with positive and negative controls. All treatments were applied once at the beginning of the experiment, and populations were monitored for 140 days under field conditions. Different exposure intervals represent post-treatment collection days. Means represent values per sample unit. Higher values indicate population growth, while lower values reflect fungal suppression of mite development and reproduction. Means of the same column followed by the same letter do not differ significantly.

Treatment	Dose (mL/100 L)	Larval Population Density (Mites/Sampling Unit) at Selected Days After Treatment (DAT)								
		0	7	14	21	28	56	84	102	140
Velifer	100		26.5 ± 2.2 a	28.5 ± 2.4 a	29.9 ± 1.9 a	32.6 ± 2.4 a	13.2 ± 2.8 a	22.2 ± 3.5 a	25.5 ± 2.9 a	16.6 ± 2.1 a
Metab	100		29.8 ± 1.3 a	33.3 ± 4.8 a	39.5 ± 3.1 a	56.1 ± 1.2 b	34.7 ± 3.2 b	53.3 ± 2.7 b	38.5 ± 1.9 a	24.6 ± 2.9 a
Botanigard	100	>10	25.6 ± 2.8 a	26.7 ± 2.9 a	31.5 ± 2.1 a	43.3 ± 3.3 a	13.9 ± 2.1 a	28.6 ± 2.4 a	32.4 ± 2.8 a	29.3 ± 2.8 a
Valmec (Positive Control)	50		22.3 ± 1.8 a	30.0 ± 0.0 a	32.8 ± 1.3 a	34.5 ± 2.8 a	11.8 ± 4.8 a	26.6 ± 3.2 a	11.3 ± 1.7 b	21.6 ± 1.8 a
dd H_2O (Negative Control)	-		53.3 ± 4.7 b	132.4 ± 33.4 b	141.3 ± 22.1 b	177.2 ± 21.1 c	136.5 ± 25.2 c	143.3 ± 10.8 c	202.5 ± 21.1 c	217.1 ± 35.1 b

Table A4. Mean (±SE) population density of protonymphs of *T. urticae* on *C. sinensis* at different intervals following foliar application of three entomopathogenic fungal treatments (Velifer, Metab, and Botanigard), along with positive and negative controls. All treatments were applied once at the beginning of the experiment, and populations were monitored for 140 days under field conditions. Different exposure intervals represent post-treatment collection days. Means represent values per sample unit. Higher values indicate population growth, while lower values reflect fungal suppression of mite development and reproduction. Means of the same column followed by the same letter do not differ significantly.

Treatment	Dose (mL/100 L)	Protonymph Population Density (Mites/Sampling Unit) at Selected Days After Treatment (DAT)								
		0	7	14	21	28	56	84	102	140
Velifer	100		12.9 ± 2.2 a	34.6 ± 2.4 a	45.6 ± 2.7 a	56.6 ± 1.2 a	50.0 ± 0.0 a	49.3 ± 1.9 a	44.4 ± 2.6 a	34.7 ± 1.5 a
Metab	100		15.6 ± 2.1 a	38.3 ± 2.9 a	49.3 ± 3.1 a	63.6 ± 2.1 a	58.4 ± 1.8 a	60.0 ± 0.0 a	54.6 ± 1.5 a	44.2 ± 2.1 b
Botanigard	100	>10	12.8 ± 1.5 a	38.1 ± 2.1 a	45.4 ± 2.5 a	58.4 ± 1.9 a	52.8 ± 2.1 a	54.4 ± 2.6 a	43.1 ± 2.2 a	35.4 ± 2.3 a
Valmec (Positive Control)	50		13.3 ± 1.9 a	28.9 ± 3.1 a	32.3 ± 2.2 b	47.2 ± 1.5 a	49.6 ± 1.9 a	45.2 ± 2.8 a	33.3 ± 1.7 b	21.1 ± 1.9 a
dd H_2O (Negative Control)	-		33.3 ± 2.8 b	47.6 ± 3.7 b	54.8 ± 2.9 c	86.6 ± 7.6 b	106.4 ± 19.8 b	125.4 ± 24.8 b	284.5 ± 32.1 c	243.6 ± 29.4 c

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