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Compatibility of the entomopathogenic fungus *Beauveria bassiana* with etoxazole, spirodiclofen and spiromesifen against *Tetranychus urticae*

Beatriz López-Manzanares [a], Elena Martínez-Villar [b], Vicente S. Marco-Mancebón [b,*], Ignacio Pérez-Moreno [b]

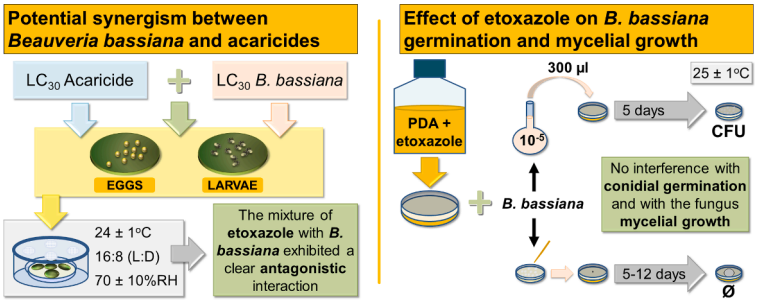
[a] Institute of Grapevine and Wine Sciences (ICVV), Spanish National Research Council (CSIC), University of La Rioja, Government of La Rioja, Ctra. LO-20 Salida 13, Finca La Grajera, Logroño, Spain

[b] Crop Protection Unit, Department of Agriculture and Food Science, Science and Technology Centre, University of La Rioja, C/Madre de Dios, 51, 26006 Logroño, Spain

HIGHLIGHTS

- *B. bassiana* exhibited an additive interaction with spirodiclofen and spiromesifen.
- The mixture *B. bassiana*-etoxazole showed a clear antagonistic interaction.
- The antagonism was not due to a decrease in conidial germination or mycelial growth.

GRAPHICAL ABSTRACT



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ABSTRACT

In the Integrated Pest Management of *Tetranychus urticae*, it is important to use microbiological control agents and acaricides with differing modes of action in order to reduce the risk of resistance, so often reported for this mite. This study evaluates the effect of etoxazole, spirodiclofen, spiromesifen and their compatibility with *Beauveria bassiana* on the eggs and larvae of *T. urticae*. For etoxazole and spirodiclofen, the regression lines from the Probit Analysis were parallel in both cases for the two developmental stages. Both acaricides were more effective on eggs than larvae (2.5 and 4.6 times, respectively). The adjusted regression lines of efficacy produced from the bioassays with spiromesifen on eggs and larvae were neither equal nor parallel. However, the confidence intervals for eggs and larvae did overlap at LC_{99}, indicating equal sensitivity in both eggs and larvae at this concentration. The mixture of etoxazole with *B. bassiana* showed clear antagonistic interaction even though this effect was the result of interference with neither conidial germination nor with the fungus mycelial growth. On the other hand, the combinations of *B. bassiana* with spirodiclofen and with spiromesifen exhibited additive interaction. The results of this research show that the effectiveness of mixtures of *B. bassiana* with both spirodiclofen and spiromesifen requires product concentrations to be maintained with no possibility of reduction. Furthermore, the antagonistic effect of the mixture of *B. bassiana* and etoxazole precludes the combined use of these acaricides in the management of the eggs and larvae of this mite.

* Corresponding author.
E-mail addresses: elena.martinez@unirioja.es (E. Martínez-Villar), vicente.marco@unirioja.es (V.S. Marco-Mancebón), ignacio.perez@unirioja.es (I. Pérez-Moreno).

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1. Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), is a significant pest in numerous food and fibre crops and ornamental plants, and is considered to be a key pest in regions with temperate climates. This mite is also considered the most polyphagous within the Tetranychidae family (Van de Vrie, 1985). Due to its elevated reproduction rate and short life cycle, this mite can reach economic thresholds quickly. The rapid increase in population densities in turn leads to the use of acaricides, which negatively affect the abundance of natural enemies. In addition, the rapid development of resistance to common chemical pesticides continues to make the control of this pest a true challenge. Thus, two key aspects, which must be kept in mind in Integrated Pest management (IPM) strategies against *T. urticae* are the protection of natural enemies and the use of selective acaricides with diverse modes of action. One basic feature of IPM is the use of biological control agents compatible with chemical pesticides, which is not only effective but also engenders fewer toxicological problems and protects the environment. In this context, we studied the compatibility of mixtures of the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin with the acaricides etoxazole, spirodiclofen and spiromesifen.

B. bassiana is a cosmopolitan, soil fungus, which has been shown to be a useful control agent for drastically reducing various populations of economically important pest species (Legaspi et al., 2000). Several authors have reported that *B. bassiana* is effective in controlling *T. urticae* populations (Islam et al., 2017; Yeşilayer, 2018). Nevertheless, this effectiveness is influenced by factors including the quantity of conidia, relative humidity and temperature (Shipp et al., 2003; Maniania et al., 2008; Shi et al., 2008; Ullah and Lim, 2015), the crop (Seyed-Talebi et al., 2014), the spider mite population (Zélé et al., 2020) and life stage (Bugeme et al., 2015), and the fungal isolate. Thus, the varied results of pathological studies using *B. bassiana* to manage *T. urticae* are not surprising. Studies employing the commercial preparation of *B. bassiana* Naturalis-L obtained mortality rates from 75% (Alves et al., 2002; Shi and Feng, 2009) to 97% (Chandler et al., 2005). On the other hand, Gatarayiha et al. (2010) obtained a mortality rate of 60% in adults, yet when Naturalis-L was mixed with silicone-based surfactants the percentages increased to 85%. The Naturalis-L LC_{50} for *T. urticae* larvae, nymphs and adults was found to be in the range of 1,949.4 to 3,184.4 conidia per mL (Sáenz-de-Cabezón Irigaray et al., 2003). However, using the same product, Shipp et al. (2003) and Castagnoli et al. (2005) found no significant effect on adult *T. urticae*.

Etoxazole is an acaricide classified as a growth inhibitor (group 10B) by the Insecticide Resistance Action Committee (IRAC, 2021) which inhibits the synthesis of chitin (Van Leeuwen et al., 2012). It is considered effective principally against tetranychid mites, but it is also useful to control aphids, grasshoppers, and moths. As an acaricide, it acts predominantly on eggs and larvae (Tirello et al., 2012), slightly less on nymphs and much less on adults (Dekeyser, 2005). To date, no studies have evaluated the compatibility of etoxazole with *B. bassiana*. In contrast, spirodiclofen and spiromesifen pertain to the group of chemicals derived from tetronic acids and are included in group 23 (Acetyl-CoA carboxylase inhibitors) which inhibit the formation of lipids and act as growth regulators (IRAC, 2021). Even though they were discovered only recently, their use has increased worldwide (Sparks and Nauen, 2015). to the fact that their mode of actions is different from the rest of the acaricides means that lipid synthesis inhibitors can be suitable to control species of pests which are resistant to other chemicals, thus facilitating Integrated Resistance Management (IRM). Spirodiclofen is highly effective against eggs and immature stages, also influences fertility and fecundity and can even kill adult death in crop pest mites (Wachendorff et al., 2002; Marcic and Ogurlic, 2006; Cheon et al., 2007). In terms of the compatibility of spirodiclofen with *B. bassiana*, Seyed-Talebi et al. (2014) found an interesting synergetic interaction for the control of *T. urticae*. On the other hand, spiromesifen is very active against juvenile stages and eggs and, although it has a strong effect on the fecundity of adults in mites and whiteflies, for example, the lethal concentrations required are very high (Nauen et al., 2005; Sato et al., 2011; Esteves Filho et al., 2013; Nicastro et al., 2013; Saryazdi et al., 2013). Very few studies have been carried out on the compatibility of *B. bassiana* with spiromesifen. Therefore, Seyed-Talebi et al. (2014) combined the LC_{25} of spiromesifen and the LC_{50} of an Iranian strain of *B. bassiana* and obtained an interesting synergistic effect against female adult *T. urticae*. However, Cuthbertson et al. (2012) observed a reduction in spore germination by this miticide for which they advise against the use of *B. bassiana* in mixtures. Dutta et al. (2016) studied in vitro incompatible *B. bassiana* with spiromesifen and Olivos Quiroz (2012), *B. bassiana* with spirodiclofen. Given that previous studies suggest the need to assess combinations of *B. bassiana* with acaricides, this study was carried out to evaluate the potential synergistic or antagonistic interactions of etoxazole, spirodiclofen and spiromesifen when mixed with *B. bassiana* against the eggs and larvae of *T. urticae*. The results of this research will enable the optimization of these combinations with a view to their effective implementation in IPM programmes.

2. Materials and methods

2.1. Colony source

The *T. urticae* individuals used in the bioassays came from a laboratory colony maintained in the Crop Protection Laboratory of the University of La Rioja. The colony was started with individuals collected from a natural population found in the ornamental plant *Impatiens walleriana* Hook. f. in the city of Logroño (Spain) in 2000. The *T. urticae* were mass-reared on green bean (*Phaseolus vulgaris* L., var. Garrafal) plants free of pesticides in a growth chamber at 24 ± 1 °C, 70 ± 10% RH, with a photoperiod of 16:8 (L:D). The bioassays were run in a separate growth chamber under the same environmental conditions as the mass rearing.

2.2. Synchronization of cohorts

Cohorts of eggs<24 h old and neonate larvae were used in the bioassays to minimize variability due to age of individuals. To obtain the egg cohorts, *T. urticae* adult females were placed on green bean leaf disks (20 mm in diameter), and allowed to lay eggs for<24 h. The adult females were then removed along with the extra eggs and the same leaf discs with appreciable numbers of eggs were used in the bioassay. To obtain the larval cohorts, the adult females were placed on the underside of leaves previously isolated on damp filter paper in Petri dishes (90 mm in diameter), and allowed to lay eggs for<24 h. Isolating the leaf on damp filter paper maintained the turgor pressure of the leaf and kept the eggs isolated until larval emergence five days later. When used in bioassays, the larvae were moved to 20 mm diameter green bean leaf disks.

2.3. Commercially available pesticide products

To evaluate the compatibility (synergistic, antagonistic or additive interactions) of each acaricide with *B. bassiana*, the LC_{30} for *T. urticae* was employed for every compound. The LC_{30} for *T. urticae* was calculated using the regression line of the Probit Analysis of each compound employed. The commercial acaricides used are listed in Table 1. In the case of *B. bassiana*, the viability of the spores was determined using the protocol by Butt and Goettel (2000). For this protocol, 30 Petri dishes (9 cm diameter) with potato-dextrose agar (PDA) culture medium was inoculated with 300 μL of a 10^{-5} dilution of Naturalis-L in sterile water (Sáenz-de-Cabezón Irigaray et al., 2003). The Petri dishes were incubated for 5 days at 25 ± 1 °C, after which the mean number of colony forming units (CFUs) was calculated along with the product concentration to be used in the bioassays (CFU/mL).

2.4. Effects of B. bassiana, etoxazole, spirodiclofen and spiromesifen on eggs and larvae

The acaricide activity on *T. urticae* eggs and larvae was determined for each compound by obtaining the Probit Analysis regression line. Green bean leaf disks containing one cohort of the pertinent life stage were placed in 9 cm diameter Petri dishes. Each plate contained three layers of filter paper on top of a layer of moist cotton to maintain leaf disk turgor pressure and keep the bioassay individuals isolated. Each leaf disk contained ten *T. urticae* individuals, eggs or larvae. The concentrations previously derived from the Probit Analysis of each phytosanitary product and the life stages are listed in Table 2. For each treatment, five replicates (acaricide and *B. bassiana*) and controls (distilled water) were made. Treatments were carried out in a Potter tower (Burkard Rickmansworth, Herts, United Kingdom) with 5 mL of treatment solution at a pressure of 50 kPa (Potter, 1952), which was equivalent to a deposit of 0.005 ± 0.0004 mL/cm^2. Considering the length of development for each life stage, data was collected over a six-day period for eggs, and five days for larvae. However, the cohorts continued to be monitored until it was observed that the individuals were unable to hatch or to moult to the next stage, respectively; the number of live and dead individuals was recorded every day for the duration of each bioassay.

Table 2
Concentrations used to obtain the Probit-log concentration regression lines fitted for *Tetranychus urticae* eggs and larvae.

Active ingredient	Concentrations	
	Eggs	Larvae
Etoxazole (mg/L)	0.007 – 0.014 – 0.028 – 0.055 – 0.110 – 0.220	0.029 – 0.065 – 0.098 – 0.147 – 0.220
Spirodiclofen (mg/L)	0.25 – 0.50 – 1.00 – 2.00 – 4.00 – 8.00	0.50 – 2.00 – 4.00 – 8.00 – 16.00
Spiromesifen (mg/L)	0.012 – 0.021 – 0.039 – 0.125 – 0.480	0.06 – 0.09 – 0.014 – 0.190 – 0.320 – 0.480
Beauveria bassiana (CFU/mL)	15,000 – 43,200 – 64,000 – 96,000 – 144,000	12,800 – 19,200 – 28,800 – 43,200 – 64,000 – 96,000

Table 3
LC_{30} for the eggs and larvae of *Tetranychus urticae*.

Active ingredient	LC_{30}	
	Eggs	Larvae
Etoxazole	0.029 mg/L	0.072 mg/L
Spirodiclofen	0.357 mg/L	1.655 mg/L
Spiromesifen	0.168 mg/L	0.126 mg/L
Beauveria bassiana	51,935 CFU/mL	30,691 CFU/mL

2.5. Potential synergism between B. Bassiana and etoxazole, spirodiclofen and spiromesifen

For each of the three chemical acaricides evaluated, the following treatments on *T. urticae* eggs and larvae were carried out: 1) *B. bassiana*; 2) Acaricide; 3) *B. bassiana* + acaricide; 4) Control (distilled water). The LC_{30} used were obtained in the corresponding bioassays and are listed in Table 3. The treatments were carried out in the Potter tower in the same manner as described above. In all treatments, five replications were made with 10 individuals per replicate. The mortality of eggs and larvae was recorded after seven days.

2.6. Effect of etoxazole on B. Bassiana germination and mycelial growth

Due to the antagonistic interaction found in the mixture of *B. bassiana* with etoxazole, the effect of this chemical on conidia germination and mycelial growth was evaluated, as important factors that affect pathogenicity. For this evaluation, the PDA was sterilized in an autoclave with a pressure of 1.02 bar at 120 °C for 45 min, allowed to cool to 45 °C before mixing with etoxazole with a concentration of LC_{30}, the concentration previously obtained for eggs (0.029 mg/L) and larvae (0.072 mg/L).

In the evaluation of the impact of etoxazole on *B. bassiana* conidia germination, twenty treatment Petri dishes were prepared with PDA + acaricide for each concentration while the control contained only PDA. Each Petri dish was inoculated with 300 μL of Naturalis-L diluted to 10^{-5} using sterile distilled water. The Petri dishes were incubated at 25 ± 1 °C for five days, then the number of CFUs was recorded.

To analyse the effect of etoxazole on mycelial growth, a sample of *B. bassiana* inoculum was obtained from cultures, which had been prepared using a 10^{-3} dilution of Naturalis-L on Petri dishes containing only PDA and allowed to grow for seven days at 25 ± 1 °C. Using the point of a sterilized toothpick, a sample of the inoculum was transferred to the centre of the Petri dishes containing PDA + etoxazole or PDA only (control) and allowed to grow for five, seven and twelve days at 25 ± 1 °C before measuring the diameter of the fungal mycelium. Fifteen replications of each treatment and control were included in the statistical analysis.

2.7. Statistical methods

Probit Analysis was using Polo Plus version 2.0 (LeOra Software, 1987) was used to estimate the LC_{30} of each of the products for *T. urticae* eggs and larvae. For all products, the parallelism was tested between each log-Probit regression line for eggs and larvae. When the log-Probit lines were parallel, the overlap was checked between confidence intervals to determine whether the regression lines were significantly different or not at a confidence level of 95%.

The χ^2 test was used to determine whether the effect produced was synergistic, additive or antagonistic when the acaricides were mixed with *B. bassiana* (Koppenhöfer and Fuzy 2008; Morales-Rodriguez and Peck 2009). The expected mortality with a mixture (M_E) was calculated using the following formula:

$$M_E = M_H + M_Q\left(1 - \frac{M_H}{100}\right)$$

Where M_H is the Abbott corrected percentage mortality (Abbott, 1925) for the fungus and M_Q is the Abbott corrected percentage mortality for the acaricide. The Abbott correct mortalities were calculated using the following formula:

$$Abbot\ \ Mortality = \frac{M_{treatment} - M_{control}}{100 - M_{control}} \times 100$$

The χ^2 values were obtained using the following formula, then

Table 1
Acaricides used in the laboratory trials.

Active ingredient	Trade name	Company	Concentration	Formulation*
Etoxazole	Borneo®	Kenogard S.A.	11.0%	SC
Spirodiclofen	Envidor®	Bayer CropScience S.L.	22.3%	SC
Spiromesifen	Oberon®	Bayer CropScience S.L.	22.9%	SC
Beauveria bassiana	Naturalis-L®	Agrichem S.A.	2.3×10^9 conidia/mL	OD

*SC: concentrated suspension; OD: oil dispersions.

compared to the expected values from the χ^2 Table for one degree of freedom and $\alpha = 0.05$.

$$\chi^2_{calculated} = \frac{(M_{QH} - M_E)^2}{M_E}$$

In this formula, M_{QH} represents the Abbott corrected percentage mortality resulting from the mixture of the acaricide and the fungus. The null hypothesis (H_o) was formulated as "there is an additive interaction between the two agents if the $\chi^2_{calculated} < \chi^2_{Table\ value}$". On the contrary, if $\chi^2_{calculated} > \chi^2_{Table\ value}$, the interaction was considered to be synergistic when M_{HQ} - M_E is positive; if M_{HQ} - M_E is negative, the interaction is considered antagonistic.

When it was necessary, means were compared using a Student's *t* test for two independent samples or a F-test of analysis of variance (ANOVA) followed by the Tukey multiple comparisons test ($\alpha = 0.05$).

3. Results

3.1. Effects of B. bassiana, etoxazole, spirodiclofen and spiromesifen on T. Urticae eggs and larvae

Table 4 lists the Probit Analysis results for the effects of *B. bassiana* and the three acaricides on *T. urticae* eggs and larvae. The data were recorded and the regression lines were calculated five days after treatment for eggs and six for larvae. The equality and parallelism tests between the Probit-log lines obtained for *B. bassiana* in eggs and larvae resulted in the rejection of the null hypothesis. The LC_{50} and LC_{99} of this entomopathogenic fungus, with the slope of the Probit-log regression line steeper for the larval stage than the egg stage, indicate greater effectiveness on larvae than on eggs.

In the case of etoxazole and spirodiclofen, the null hypothesis was rejected for equality and accepted for parallelism. The egg stage was the most sensitive for both acaricides. The relative potency values were 2.5 for etoxazole and 4.6 for spirodiclofen; that is, the effect on eggs is 2.5 and 4.6 times greater than in larvae respectively for both compounds. Finally, the regression lines obtained for the effect of spiromesifen on eggs and larvae were neither equal nor parallel. However, the LC_{50} and LC_{99} did not present significant differences between the effect on eggs and larvae, due to the overlap of the confidence intervals in both lethal concentrations.

3.2. Potential synergy between B. Bassiana and etoxazole, spirodiclofen and spiromesifen

The combination of *B. bassiana* and etoxazole resulted in an interaction considered antagonistic on *T. urticae* eggs (Table 5) and larvae (Table 6). The mixtures of *B. bassiana* with spirodiclofen and *B. bassiana* with spiromesifen instead both resulted in additive interactions, on both mite eggs and larvae (Tables 5 and 6, respectively).

3.3. Effects of etoxazole on B. Bassiana germination and mycelial growth.

There were no significant differences between the number of CFUs in the control dishes (30.47 ± 11.32) and in the dishes treated with etoxazole LC_{30} for *T. urticae* eggs (32.33 ± 9.59) (t = -0.49; P = 0.6299) nor for the LC_{30} for larvae (34.40 ± 7.10) (t = -1.14; P = 0.2641). Thus, etoxazole is compatible with the conidia germination of *B. bassiana*. In addition, the LC_{30} for eggs caused no significant reduction in mycelial growth in this fungus at five days (t = 0.98; P = 0.3387) or at seven days (t = 0.66; P = 0.5140). However, at 12 days, the mycelial diameter was 3.16% lower in the etoxazole replications (t = 5.20; P < 0.0001) than in the control (Table 7). Similarly, the LC_{30} for etoxazole on larvae caused no significant reduction in mycelial growth in this fungus at 5 days (t = 0.27; P = 0.7924, or at 7 days (t = 0.50; P = 0.1474). However, at 12 days, the mycelial diameter was 7.19% lower in the etoxazole treatment (t = 2.83; P = 0.0096) than in the control (Table 7).

4. Discussion

This study investigates the effectiveness and compatibility of several acaricides with *B. bassiana,* with a view to incorporating the use of such mixtures into the Integrated Pest Management of the two-spotted spider mite.

Considering *B. bassiana* alone, the *T. urticae* eggs were less susceptible than larvae (LC_{50} = 1.12 × 10^5 CFU/mL and 48,261 CFU/mL, respectively). This lower susceptibility of the eggs agrees with the observations of Sáenz-de-Cabezón Irigaray et al. (2003) and could be explained by the topography of the chorion, which can make it difficult for conidia to establish (Leger et al. 1991). Due to the important influence that the composition of substrate has on this fungus, the nutrients found on the egg surface could play a key role in the germination and development of *B. bassiana* (Bidochka and Khachatourians 1992; Napolitano and Juárez 1997).

Etoxazole, on the other hand, is an acaricide that acts mainly on eggs and larvae (Tirello et al. 2012), which explains why the LC_{50} obtained in this study are considerably inferior to those found for adults by other authors such as Nicastro et al. (2013). The results show lower susceptibility of *T. urticae* larvae than eggs. The results for the LC_{50} calculated for eggs are within the same range found by Tirello et al. (2012) in various Italian populations. There are no previous studies that evaluate the compatibility of etoxazole and *B. bassiana*. The mixture resulted in an antagonistic interaction in eggs and larvae. This antagonistic effect could not be attributed to either the conidial germination or the mycelial growth of the fungus, given that there was no effect seen aside from a slight reduction in mycelial diameter in the longer term.

Spirodiclofen is another chemical that is considered exceptionally effective against eggs and immature stages of phytophagous mites. It has less effect on adult females even though it does seriously affect fecundity and fertility, provoking death in a very few days (Wachendorff et al.

Table 4
Parameters of the Probit-log concentration regression lines fitted for *Tetranychus urticae* eggs and larvae, treated with *Beauveria bassiana*, etoxazole, spirodiclofen and spiromesifen.

Active ingredients	Development stage	Probit equation	χ^2 calculated (df) *	LC_{10} (fl, 95%)**	LC_{50} (fl, 95%)**	LC_{99} (fl, 95%)**
B. bassiana	Eggs	y = -2.94 + 1.57 x	0.397 (3)	17,130 (5,065–28,311)	1.12 × 10^5 (84,115–1.85 × 10^5)	3.38 × 10^6 (1.01 × 10^6– 7.66 × 10^7)
	Larvae	y = -7.49 + 2.67 x	1.832 (3)	15,965 (11,672–19,862)	48,261 (41,267–57,668)	3.59 × 10^5 (2.34 × 10^5– 6.96 × 10^5)
Etoxazole	Eggs	y = 10.46 + 3.88 x	1.979 (4)	0.018(0.013–0.023)	0.039 (0.033–0.046)	0.156 (0.116–0.255)
	Larvae	y = 8.72 + 3.72 x	0.924 (3)	0.045 (0.034–0.054)	0.100 (0.088–0.112)	0.421 (0.320–0.641)
Spirodiclofen	Eggs	y = 5.55 + 2.40 x	1.070 (4)	0.173 (0.099–0.247)	0.590 (0.460–0.724)	5.484 (3.642–10.424)
	Larvae	y = 3.94 + 2.46 x	6.392 (3)	0.815 (0.158–1.499)	2.704 (1.454–4.268)	23.829 (11.182–208.036)
Spiromesifen	Eggs	y = 7.21 + 3.53 x	4.069 (3)	0.103 (0.029–0.158)	0.237 (0.152–0.388)	1.080 (0.560–11.251)
	Larvae	y = 9.07 + 5.12 x	7.041 (4)	0.090 (0.062–0.111)	0.160 (0.134–0.192)	0.455 (0.332–0.853)

*df degree of freedom; **fl fiducial limits.

Table 5
Effect of combinations of *Beauveria bassiana* and etoxazole, spirodiclofen and spiromesifen on *Tetranychus urticae* egg mortality.

Treatment	Mortality (Mean ± SE)*	$\chi^2_{calculated}$ (α = 0.05, df = 1)	$\chi^2_{Table\ value}$ (α = 0.05, df = 1)	D**	Statistics
Control	7.14 ± 1.84 a				
B. bassiana	34.29 ± 6.85b				
Etoxazole	32.86 ± 3.60b				
B. bassiana + Etoxazole	34.29 ± 6.12b	7.866	3.841	−19.60	$F_{3,24} = 7.08$; $P < 0.0014$
Control	7.14 ± 1.84 a				
B. bassiana	34.29 ± 6.85 bc				
Spirodiclofen	24.29 ± 6.12 ab				
B. bassiana + Spirodiclofen	51.43 ± 7.38c	0.689	3.841	–	$F_{3,24} = 9.67$; $P < 0.0000$
Control	7.14 ± 1.84 a				
B. bassiana	34.29 ± 6.85 bc				
Spiromesifen	20.00 ± 7.87 ab				
B. bassiana + Spiromesifen	41.43 ± 6.70c	0.114	3.841	–	$F_{3,24} = 5.92$; $P < 0.0036$

*Means within a B. bassiana – acaricide combination followed by the same letter are not significantly different at the 5% level (ANOVA and LSD). **D = (MHQ - ME) < 0 a significant antagonistic interaction.

Table 6
Effect of combinations of *Beauveria bassiana* and etoxazole, spirodiclofen and spiromesifen on *Tetranychus urticae* larva mortality.

Treatment	Mortality (Mean ± SE)*	$\chi^2_{calculated}$ (α = 0.05, df = 1)	$\chi^2_{Table\ value}$ (α = 0.05, df = 1)	D**	Statistics
Control	0.00 ± 0.00 a				
B. bassiana	27.14 ± 5.22b				
Etoxazole	38.57 ± 8.00b				
B. bassiana + Etoxazole	31.43 ± 7.69b	10.267	3.841	–23.82	$F_{3.24} = 7.57$; $P < 0.0010$
Control	0.00 ± 0.00 a				
B. bassiana	27.14 ± 5.22c				
Spirodiclofen	12.86 ± 2.86b				
B. bassiana + Spirodiclofen	42.86 ± 5.65 d	1.103	3.841	–	$F_{3.24} = 20.24$; $P < 0.0000$
Control	0.00 ± 0.00 a				
B. bassiana	27.14 ± 5.22b				
Spiromesifen	12.86 ± 3.60 ab				
B. bassiana + Spiromesifen	45.71 ± 8.12c	2.320	3.841	–	$F_{3.24} = 14.51$; $P < 0.0000$

*Means within a *B. bassiana* – acaricide combination followed by the same letter are not significantly different at the 5% level (ANOVA and LSD). **D = (M_{HQ} - M_E) < 0 a significant antagonistic interaction.

Table 7
Radial mycelial in vitro growth of *Beauveria bassiana* (Mean ± SE) at 5, 7 and 12 days after inoculation with addition of etoxazole at a concentration to the LC_{30} for eggs and larvae of *Tetranychus urticae*.

Treatment	Mycelial growth (cm)			% inhibition		
	5 days	7 days	12 days	5 days	7 days	12 days
Control (*B. bassiana* alone)	1.51 ± 0.05	2.48 ± 0.06	4.75 ± 0.10			
B. bassiana + Etoxazole (0.029 mg/L)	1.49 ± 0.03	2.47 ± 0.07	4.60 ± 0.00*	–	–	3.16
B. bassiana + Etoxazole (0.072 mg/L)	1.50 ± 0.10	2.43 ± 0.12	4.41 ± 0.41*	–	–	7.19

*Means significantly different in regard to the control (*t*-Student; α = 0.05).

2002; Marcic and Ogurlic 2006; Cheon et al. 2007; Seyed-Talebi et al. 2014). Marcic and Ogurlic (2006) found larvae to be more sensitive than eggs, which is in contrast to the findings in our bioassays that showed that the LC_{50} for larvae was much higher than for eggs (2.70 g/mL and 0.59 g/mL, respectively). Regarding the lethal concentrations of spirodiclofen for *T. urticae* eggs, previous studies show similar values to those obtained in our study (Rauch and Nauen, 2003; Tirello et al. 2012; Saryazdi et al., 2013). In relation to the compatibility of *B. bassiana* with spirodiclofen, Seyed-Talebi et al. (2014) found the mixture produced a synergistic interaction in adults. In the current research, however, the effect was additive for both eggs and larvae. From the point of view of the incorporation of spirodiclofen in IPM programmes, the demography of the population must be kept in mind. The majority—-90% of the individuals in stable natural populations of this mite—consists of eggs and immature stages (Carey 1982; Helle and Sabelis 1985) which are precisely those most sensitive to spirodiclofen. In addition to its different mode of action based on the inhibition of lipid synthesis, this acaricide has the advantage of effectiveness in resistant populations of mites (Nauen et al. 2000; Elbert et al. 2002; Dekeyser 2005). The only negative aspect of spirodiclofen is its poor efficacy in adults; however, keeping in mind the synergistic interaction found by Seyed-Talebi et al. (2014) with *B. bassiana*, this acaricide could be an interesting option for mixtures to treat *T. urticae* populations. In various European countries, spirodiclofen at 96 mg/L is an adequate concentration to control populations of *T. urticae* (Elbert et al. 2002). Field trials and laboratory bioassays employing concentrations between 48 and 96 mg/L, and or applications of 240 g/ha (Wolf and Schnorbach 2002; de Maeyer et al. 2002; Hardman et al. 2003; Reis et al. 2006; Cheon et al. 2007) have had an effect on survival and fecundity in various species of predatory mites. These findings support the ideal strategies, which use lower concentrations permitting the integration of biological control agents.

In respect to spiromesifen, *T. urticae* larvae and eggs showed similar susceptibilities. This acaricide acts best on eggs and larvae, so the lethal concentrations for adults are higher (Nauen et al. 2005; Sato et al. 2011; Esteves Filho et al. 2013; Nicastro et al. 2013; Saryazdi et al., 2013). There have been very few studies on the compatibility of spiromesifen with *B. bassiana*. Cuthbertson et al. (2012) detected a reduction in fungal spore germination; thus, they advise against mixing the two. However, upon mixing these products in our study, we found an additive interaction in both larvae and eggs. There does not appear to be any advantage to using this combination, though neither is there any reduction in effectiveness.

In summary, the results of this research show that the antagonistic effect between *B. bassiana* and etoxazole precludes their combined use in the management of eggs and larvae of *T. urticae*. In contrast, *B. bassiana*

can be combined with both spirodiclofen and spiromesifen for improved treatment against *T. urticae*. However, results show that the effectiveness in treating these stages of the mite requires product concentrations to be maintained without reduction.

5. Author statement

The authors of the manuscript are:

Beatriz López-Manzanares (BLM); Elena Martínez-Villar (EMV); Vicente S. Marco-Mancebón (VSMM) and Ignacio Pérez-Moreno (IPM).

- BLM, VSMM and IPM conceived and designed research.
- BLM and EMV conducted experiments and acquired the data.
- BLM, VSMM and IPM analyzed data.
- BLM, VSMM and IPM wrote the manuscript.

- BLM, EMV, VSMM and IPM revising the manuscript critically for important intellectual content. Finally, all the authors read and approved the manuscript.
- BLM, EMV, VSMM and IPM participated in the article submission and review process.

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