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The entomopathogenic fungus *Beauveria bassiana* and its compatibility with triflumuron: effects on the twospotted spider mite *Tetranychus urticae*

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Abstract

Laboratory studies were conducted to determine the effects of the mycoinsecticide Naturalis-L (*Beauveria bassiana* conidial formulation) on the twospotted spider mite *Tetranychus urticae*. Compatibility of *B. bassiana* with triflumuron (benzoylphenyl urea typically used as an insecticide, but also with acaricide effects), was also investigated in order to incorporate both in the control of this pest. For each juvenile stage, 180–22,800 viable conidia/mL on deutonymphs, 380–12,160 viable conidia/mL on protonymphs, and 712–7480 viable conidia/mL on larvae were evaluated. For the adult stage, the concentrations ranged from 213 to 54,720 viable conidia/mL. The mortality data used in the analysis were those accumulated after 5 days of treatment for deutonymphs and protonymphs, 7 days for larvae and 9 days for adults. The lethal concentration to kill 50% (LC_{50}) for the juvenile stages was 3184 viable conidia/mL (their probit-log concentration regression lines were the same), and 1949 viable conidia/mL for adults. No significant differences in mortality were observed among egg age classes (24-, 48-, 72-, and 96-h-old eggs) at the tested concentrations (1400–22,800 viable conidia/mL). When *B. bassiana* was combined with 0.25 g Alsystin (25% triflumuron as a wettable powder)/L, mite egg mortality decreased significantly. Triflumuron reduced mycelial growth but not conidial germination of *B. bassiana*. This fungus is a possible candidate to be included in integrated pest management programs with triflumuron of *T. urticae*. In such programs, the possible antagonist effects of triflumuron should be considered.

Keywords: Entomopathogenic fungus; *Beauveria bassiana*; Spider mite; *Tetranychus urticae*; Triflumuron; Benzoylphenyl ureas; Compatibility

1. Introduction

The twospotted spider mite, *Tetranychus urticae* Koch, has been recorded on more than 150 hosts of some economic value throughout the world (Jeppson et al., 1975). It has recently become a serious problem because of the continuous use of pesticides (Young-Joon et al., 1993) resulting in resistance among mite population. Thus, there is a need to find alternative control measures to suppress mite populations.

The entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin, is widely distributed in nature (St. Leger et al., 1992) and has the potential to control over 70 insect pest species (Aleshina, 1980). This fungus is usually applied as a conidial spray. It has been tested in the laboratory and field against numerous insect pests such as thrips, whiteflies, and aphids (Legaspi et al., 2000). Moreover, this fungus also appears to be innocuous to most non-target organisms (Goettel et al., 1990). Studies of pathogenicity on mites have received much less attention, despite the large number of pest species. The small size of mites makes disease diagnosis difficult. Mites, however, can often be reared easily in large quantities, allowing for detailed epizootiological studies (Van der Geest et al., 2000).

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Beauveria bassiana has been cited as a host on various mites. Peña et al. (1996) demonstrated the pathogenicity of *B. bassiana* on the broad mite *Polyphagotarsonemus latus* (Banks). Dresner (1949) treated *T. urticae* with a dust containing 0.5% conidia of this fungus in the field and obtained a mortality of 71%. However, in the laboratory, Andreeva and Shternshis (1995) tested *B. bassiana* against *T. urticae* with poor results, and Tamai et al. (1999) tested the pathogenicity of one *B. bassiana* isolate against the twospotted spider mite with mortality no higher than 50%. Another *B. bassiana* isolate was tested against *T. urticae* in chrysanthemum in a semi-field experiment with results that were better than those obtained with a chemical pesticide (Alves et al., 1998). The compatibility of *B. bassiana* with the predator mite, *Neoseiulus cucumeris* (Oudemans), was studied in the laboratory and glasshouse on cucumbers with no adverse effects on the predator mite populations (Jacobson et al., 2001).

Combined application of mycoinsecticides and synthetic chemical pesticides is an attractive approach, because the fungus and chemical insecticide may act synergistically allowing the use of lower concentrations and decreasing the likelihood of resistance to either agent (Boman, 1980). Triflumuron, a benzoylphenyl urea (BPU) that inhibits chitin synthesis in insects, could a priori be a "true synergist" acting as a general stressor, making the insect more susceptible to disease, and facilitating entry of pathogenic fungi into insects by weakening the insect's cuticle. For example, lepidopteran larvae that had molted after one treatment with BPU and treated with *Metarhizium anisopline* (Metschnikoff) conidia were more susceptible to this fungus than non-treated larvae (Hassan and Charnley, 1989). The compatibility of several insecticides with *B. bassiana* had been studied by different authors in various insect pests with different results, such as, no inhibition of conidial germination (Anderson and Roberts, 1983; Anderson et al., 1989), reduction in infectivity (Olson and Oetting, 1999), or increase of infectivity (Anderson et al., 1989; Hassan and Charnley, 1989; Reuter et al., 1995; Delgado et al., 1999).

In this paper, we describe studies to determine the acaricidal activity of one commercial formulation of *B. bassiana* called Naturalis-L on *T. urticae* life stages and to evaluate its compatibility and activity when applied with triflumuron in laboratory bioassays. Finally, we discuss the potential use of *B. bassiana* in the management of *T. urticae* and the compatibility of both agents as potential acaricides.

2. Materials and methods

2.1. Mite and pesticides

Tetranychus urticae population used for the bioassays was reared in the laboratory on green bean plants (*Phaseolus vulgaris* Linnaeus, var. Garrafal) at $27 \pm 1\,^{\circ}C$, $70 \pm 10\%$ relative humidity, and 16:8 (L:D) photoperiod. The tetranychid mite population consisted of the following stages: egg, larva, protonymph, deutonymph, and adult. All stages were tested against the fungus.

The mycopesticide used was the commercial preparation of *B. bassiana*, Naturalis-L (2.3×10^9 conidia/mL) sold in Spain by Agrichem Sociedad Anónima. As suggested by Butt and Goettel (2000), the viability of the inoculum was determined close to the application times. *B. bassiana* conidia were prepared for testing by suspending them in sterile water ($1/10^5$ mL of Naturalis-L were diluted in 1 L of water first, diluting $1/10^3$ mL in 1 L, and finally, $1/10^2$ mL of the new suspension in another liter) and 0.3 mL of this suspension was pipetted on petri dishes with potato dextrose agar (PDA) medium. The viability of the *B. bassiana* conidia was validated by germination on this medium. Fifteen replicates were made. The plates were held at 27 °C and counts of colony forming units (CFU) were made after 5 days. The results indicated that the number of viable conidia was of $3.8 \times 10^6 \pm 2.7 \times 10^5$ conidia/mL of Naturalis-L (0.17% viability). Triflumuron was obtained as Alsystin (25% compound active, wettable powder) from Bayer Hispania (Spain).

2.2. Synchronous cohorts

To synchronize the developmental stages, adult female mites taken from the colony were placed on single green bean leaves on wet filter paper, inside a petri dish (90-mm diameter), allowing them to lay eggs for 12 h. The eclosed mites from these eggs were used for the bioassays.

2.3. Effects of Naturalis-L on T. urticae

Pesticide applications (Naturalis-L with distilled water) were made using a hand sprayer (Butt and Goettel, 2000). The amount of compound applied was measured previously and it was of $9.6 \pm 0.83\,\mu L/cm^2$.

Each bioassay consisted of five replicates per concentration and controls treated with distilled water alone. Each replicate consisted of 10 larvae, protonymphs, deutonymphs or adults on a green bean leaf disk, 20-mm diameter. Each leaf disk was placed on wet filter paper in 90-mm diameter petri dishes before the spray application. The treated leaf disks were air-dried for 5 min, and then the petri dishes were covered with their tops which had two holes of 6-mm diameter. Petri dishes were placed in a growth chamber at $27 \pm 0.2\,^{\circ}C$, $70 \pm 5\%$ relative humidity and 16:8 (L:D) photoperiod.

The concentrations (viable conidia/mL) were selected to establish bioassay protocols. For each juvenile mite stage, the concentration ranged from 180 to 22,800

viable conidia/mL for deutonymphs, 380 to 12,160 viable conidia/mL for protonymphs, and 712 to 7480 viable conidia/mL for larvae. For the adult stage, the concentration ranged from 213 to 54,720 viable conidia/mL. After the treatments, the number of dead individuals was counted daily. The mortality data used in the analysis were those accumulated after 5 days of treatment for deutonymphs and protonymphs, 7 days for larvae, and 9 days for adults. After incubation, growth and sporulation of *B. bassiana* on dead mites were observed. The experiments were repeated at least four times to ensure reproducibility of the results.

2.4. Ovicidal effects of B. bassiana on T. urticae

In order to evaluate ovicidal effects, two adult females of *T. urticae* were placed on a green bean leaf disk to allow them to lay eggs for 12 h. Egg groups of different ages classes (0–24, 24–48, 48–72, and 72–96 h) were treated with Naturalis-L using a hand sprayer at fungal concentrations ranging from 1400 to 22,800 viable conidia/mL. Five replicates per concentration and a water control were made. Egg hatch was recorded 6 days after treatment. The experiment was repeated once to ensure reproducibility of the results.

2.5. Potential synergism between B. bassiana and triflumuron

To evaluate the potential synergism between *B. bassiana* and triflumuron when applied together, several treatments were done on larvae as above: (1) Naturalis-L, (2) Alsystin, (3) Naturalis-L + Alsystin, and (4) control with distilled water only. The concentrations were 855 viable conidia/mL) and 0.25 g Alsystin/L (62.5 ppm triflumuron). These concentrations are close to the LC_{20} for Naturalis-L and LC_{40} for Alsystin on the larval stage. Ten larvae were treated per replicate and five replicates were done on each treatment. The mortality of individuals was counted 8 days after treatment. The experiment was repeated once to ensure reproducibility of the results.

2.6. Effects of triflumuron on B. bassiana germination and mycelial growth

Autoclaved PDA medium, cooled to 45 °C, was thoroughly mixed with Alsystin (0.25 g/L; i.e., 62.5 ppm triflumuron). Twenty mL of the mixture was poured into petri dishes (90-mm diameter) and cooled. The viability of the *B. bassiana* conidia was validated by germination on this medium. Fifteen replicates were made for the treatment and the control (PDA medium alone). The plates were held at 27 °C and counts of CFU were made after 5 days.

To evaluate the effect of triflumuron on *B. bassiana* mycelial growth, a similar method described by Todorova et al. (1998) was followed. Inoculum of *B. bassiana* was produced in petri dishes on PDA medium for 15 days at 27 °C. A small plug (3-mm deep, 7-mm diameter) of PDA with *B. bassiana*, was deposited in the center of each petri dish containing PDA only (control) and the mixture of PDA + triflumuron. The dishes were incubated at 27 °C, and the linear growth in excess of the plugs was measured on the 7th, 10th, and 14th days following the treatment. Growth was measured with a ruler at the four cardinal points from the plug, and the mean value was used in the statistical tests. In this case, 20 replicates were used for the treatment and the control.

2.7. Statistical methods

Estimates of LC_{50} and LC_{90} values for different stage mortalities and their 95% fiducial limits were obtained using the Polo program (Russell et al., 1977) based on Finney (1971). A parallelism test was performed according to the relative potency estimation method. The criterion of overlapping fiducial limits of the LC_{50} and LC_{90} was used to establish whether lines were significantly different or not at the 5% level.

The ovicidal effects of *B. bassiana*, the effects of triflumuron on mycelial growth, and data about potential synergism between *B. bassiana* and triflumuron were tested by one-way analysis of variance (ANOVA) and means were separated by the LSD multiple range test ($p = 0.05$). The effect of triflumuron on *B. bassiana* germination was tested using Student's *t* test. The SPSS program was used in all cases (SPSS, 1999).

3. Results

3.1. Effects of Naturalis-L on T. urticae

Table 1 shows the log dose-probit regression lines obtained for immature and adult stages of *T. urticae*. For the juvenile stages, hypothesis of equality (slopes and intercepts are the same) was accepted ($\chi^2 = 8.366$; $df = 13$). Table 1 also shows the LC_{50} and LC_{90} obtained for each developmental stage.

3.2. Ovicidal effects of B. Bassiana on T. urticae

No significant differences were observed among the different egg age classes at all tested concentrations. Nevertheless, the concentration level influenced the mortality of egg in each age classes (Fig. 1).

3.3. Potential synergism between B. bassiana and triflumuron

When *B. bassiana* and triflumuron were sprayed in combination, the effect on the twospotted spider mite

Table 1
Parameters of the probit-log dose regression lines fitted for *Tetranychus urticae* larvae, protonymphs, deutonymphs, and adults, sprayed with Naturalis-L (commercial preparation of *Beauveria bassiana*)

Developmental stage	Slope ± SE	Intercept ± SE	χ^2	g	LC_{50} (viable conidia/mL) (fiducial limits 95%)	LC_{90} (viable conidia/mL) (fiducial limits 95%)
Larvae and nymphs[a]	1.377 ± 0.124	5.05 ± 0.00	0.64	0.031	3184.4 (2, 629.6, 3, 873.6)	27,147.2 (18, 578.2, 45, 824.2)
Adults	0.703 ± 0.123	5.204 ± 0.124	0.08	0.117	1949.4 (695.4, 4, 313)	129,496.4 (43, 399.8, 921, 899)

[a] Larvae, protonymph, and deutonymph have the same line.

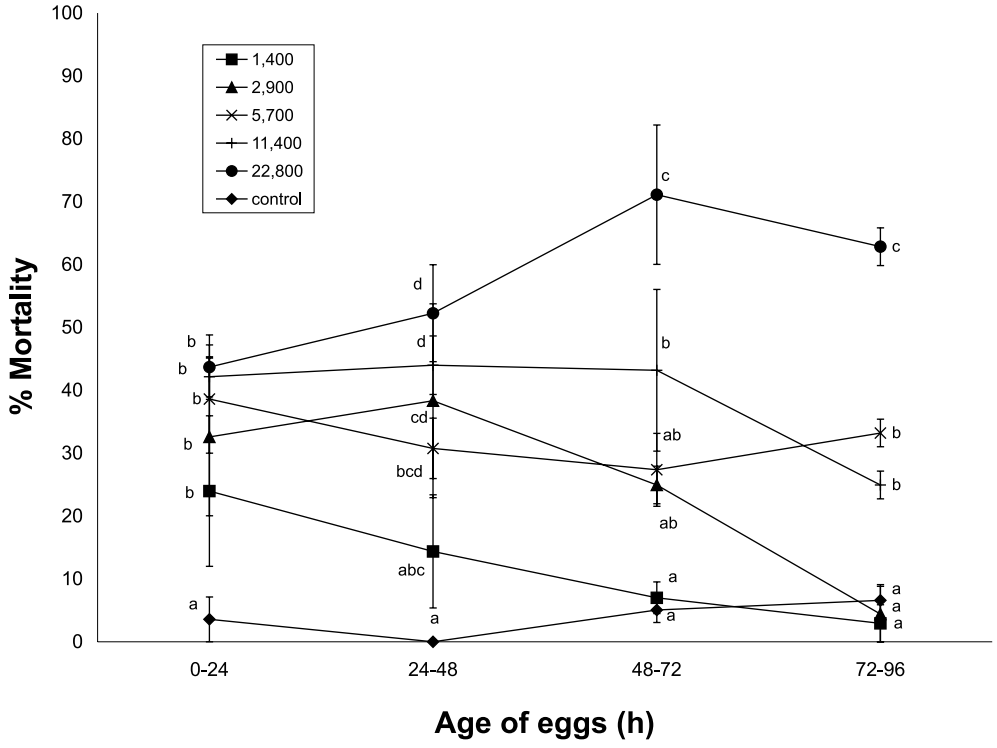

Fig. 1. Influence of *Tetranychus urticae* egg's age on the direct contact ovicidal activity of Naturalis-L (commercial formulation of *Beauveria bassiana*) at different concentrations (indicated in the legend, as viable conidia/mL). Within each age class, the data followed by the same letter are not significantly different at the 5% level (ANOVA and LSD). The vertical lines indicate the SEM.

newly emerged larvae was significantly less ($F = 10.708$, df $= 3, 16$, $p < 0.01$) than when *B. bassiana* was used alone. No significant differences were observed between the combined treatment with both compounds and triflumuron alone (Table 2). These results indicate that triflumuron had an antagonist effect on *B. bassiana*.

3.4. Effects of triflumuron on B. bassiana germination and mycelial growth

Triflumuron was compatible with *B. bassiana* germination. No significant differences were detected in CFU between PDA + triflumuron (137.2 ± 7.5) and control media (127.7 ± 9.1) (t Student; $t = 0.814$, df $= 28$, $p = 0.423$).

Fig. 2 shows the mycelial growth on PDA + triflumuron and on control media. Triflumuron significantly inhibited the linear growth of *B. bassiana* on PDA (7 days after treatment: ANOVA, $F = 212.399$, df $= 1, 38$, $p = 0.0001$; 10 days after the treatment: ANOVA, $F = 375.872$, df $= 1, 38$, $p = 0.0001$; and 14 days after

Table 2
Mortality of *Tetranychus urticae* neonate larvae sprayed with Naturalis-L (commercial preparation of *Beauveria bassiana*), Alsystin (25% triflumuron), and Naturalis-L + Alsystin 8 days after treatment

Treatment	Percentage mortality ± SE[a]
Control	12 ± 3.7a
Naturalis-L	56 ± 5.1c
Alsystin	26 ± 5.1ab
Naturalis-L + Alsystin	38 ± 8.0b

[a] Mean ± SE. Means of mortality percentages followed by the same letter do not differ significantly at the 5% level (ANOVA and LSD).

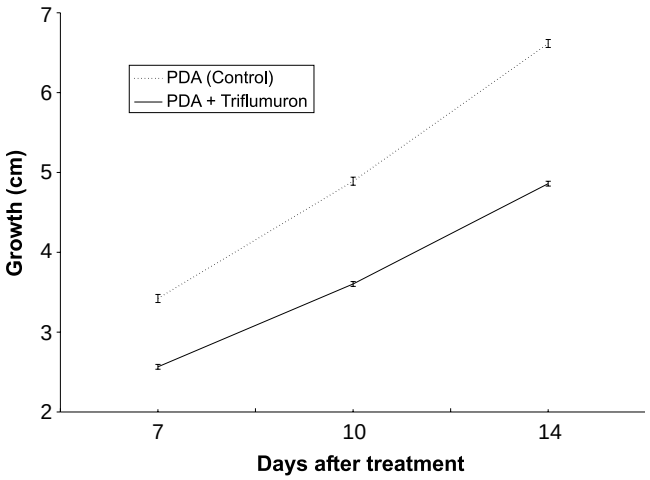

Fig. 2. Effect of triflumuron at the concentration of 62.5 ppm on the linear growth of *Beauveria bassiana* on potato dextrose agar (PDA). The vertical lines indicate the SEM.

the treatment: ANOVA, $F = 755.608$, df $= 1, 38$, $p = 0.0001$).

4. Discussion

The formulated conidia of *B. bassiana*, Naturalis-L, a commercial mycoinsecticide, can be effective for the control of the twospotted spider mite. Previous studies had showed mixed results. Dresner (1949) and Alves et al. (1998) observed that *B. bassiana* controlled this mite, whereas Andreeva and Shternshis (1995) obtained poor results. In our research, 1949 viable conidia/mL was the LC_{50} for adults and 3184 viable conidia/mL for the juvenile stages. The differences in these results could be due to the different formulations employed. That is, studies carried out by Kaaya and Hassan (2000) with the same strain, showed that the oil formulation induced a higher mortality of nymphs and adults of tick species than the aqueous formulation. On the other hand, the use of different strains of *B. bassiana* could have also influenced the results.

Mortality of eggs treated with *B. bassiana* did not differ significantly among the different egg age classes (Fig. 1). Eggs were more resistant to the fungus than the other developmental stages. The reasons for these results could be that the egg shell surface is not adequate for establishment of conidia because of its topography (St. Leger et al., 1991) and/or to the development of *B. bassiana* because of the lack of nutrients (lipids) that are necessary for germination and growth of the fungus (Bidochka and Khachatourians, 1992; Napolitano and Juarez, 1997).

No inhibition of *B. bassiana* germination by triflumuron was detected. The number of CFUs in treated and non-treated medium was not significantly different. Anderson and Roberts (1983) and Anderson et al. (1989) obtained similar results with *B. bassiana* treated with diflubenzuron and triflumuron, respectively. In examining the effects of a chemical on the fungus in vitro, it is very important to consider the formulations used for the agents. For example, the additives in the formulation of the diflubenzuron significantly affected fungal growth compared to the active ingredient on *B. bassiana* conididal germination (Anderson and Roberts, 1983). Generally, the wettable powder and flowable formulations caused no inhibition and often increased colony counts, whereas the emulsionable concentrate formulation frequently inhibited *B. bassiana* gemination (Anderson et al., 1989). On the other hand, conidia in a wettable powder are physically more accessible to the BPUs, than conidia in a Naturalis-L formulation diluted in water. These physical considerations must be taken into account. Olson and Oetting (1999) recommended that both the fungus and the diflubenzuron should not be mixed in the same tank, and when using diflubenzuron, it must dry on the foliage before *B. bassiana* can be applied.

Mycelial growth showed significant differences between treated and non-treated medium. Triflumuron significantly decreased mycelial growth. A similar result was obtained by Dmoch (1988) with the mushroom *Agaricus bisporus* (Lange) Pilat and Alsystin. It is possible that triflumuron affects the chitin synthesis of *B. bassiana* and also inhibits DNA synthesis (Binnington and Retnakaran, 1991).

Different results are recorded by different authors with *B. bassiana* and benzoylphenyl ureas. Our results are in agreement with Olson and Oetting (1999) who showed that diflubenzuron interfered with the infectivity of *B. bassiana* on the aphid *Myzus persicae* (Sulzer), reducing it up to 50%. The reduction in the mycelial growth could be responsible for this antagonistic effect observed when both types of pesticides are used simultaneously. Nevertheless, Anderson et al. (1989) found that the prevalence of mycoses from triflumuron combinations with *B. bassiana* was greater on the Colorado potato beetle than mycoses from *B. bassiana* alone. Delgado et al. (1999) obtained additive effects of *B. bassiana* and diflubenzuron against the Savanna grasshopper complex in the field, and Reuter et al. (1995) showed that diflubenzuron synergizes *Beauveria,* also in grasshoppers, increasing the speed of mortality in laboratory experiments. Probably, there may be other mechanisms (difference in the inhibition of chitin synthesis by BPUs), at work in the observed instances of synergism from simultaneous use of *B. bassiana* and BPUs. That is, some sort of interference in humoral melanization reactions may occur, because there is no evidence that these compounds degrade the cuticle so as to make fungal penetration easier. However, inhibition of chitin synthesis could be responsible for enhancement

of the fungal infection. Thus, Hassan and Charnley (1989) showed that *Manduca sexta* (L.) larvae treated with diflubenzuron followed by the fungus after the insect molted, were more susceptible to fungal infestation.

Our results indicate that *B. bassiana* could be a possible candidate to be included in integrated pest management programs of *T. urticae*. In these programs, the possible antagonist effects of other pesticides, e.g., triflumuron, on the fungus needs to be taken into consideration.

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