An agricultural detergent as co-adjuvant for entomopathogenic fungi and chlorpyrifos to control *Pseudococcus viburni* (Hemiptera: Pseudococcidae)

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Abstract

The agricultural detergent TS-2035® was evaluated using a Potter tower in the laboratory to expose *Pseudococcus viburni* (Signoret) (Hemiptera: Pseudococcidae) females to a non-lethal concentration (0.001% v/v) as a co-adjuvant of formulations containing the entomopathogenic fungi *Beauveria bassiana* Vuillemin (Cordycipitaceae) or *Metarhizium anisopliae* Sorokin (Clavicipitaceae), or the organophosphate insecticide chlorpyrifos. At that concentration, TS-2035 did not significantly remove the epicuticle wax of the pseudococcids, nor affect the pH of the solution. Adding the detergent to the *M. anisopliae* and chlorpyrifos formulations significantly reduced the LC_{50} of those solutions at 24, 72, and 144 h post treatment. For *B. bassiana*, the detergent significantly decreased the LC_{50} of that product only at 72 h post treatment. Fungal solutions with detergent did not affect the conidial germination of the entomopathogenic fungi. Our results found that mixing *B. bassiana*, *M. anisopliae*, or chlorpyrifos formulations with TS-2035 at 0.001% v/v did not control *P. viburni* by removal of their epicuticular wax, but still contributed significantly to mortality.

Key Words: *Beauveria bassiana*; insect integument; integrated pest management; *Metarhizium anisopliae*; toxicology

Resumen

El detergente agrícola TS-2035® fue evaluado utilizando una torre Potter en el laboratorio para exponer las hembras de *Pseudococcus viburni* (Signoret) (Hemiptera: Pseudococcidae) a una concentración no letal (0,001% v/v) como coadyuvante de formulaciones que contienen hongos entomopatógenos *Beauveria bassiana* Vuillemin (Cordycipitaceae) o *Metarhizium anisopliae* Sorokin (Clavicipitaceae), o el insecticida organofosforado clorpirifos. A esa concentración, TS-2035 no eliminó significativamente la cera epicutícula de los pseudococcidos, ni afectó el pH de la solución. Al agregar el detergente a las formulaciones de *M. anisopliae* y clorpirifos redujo significativamente la CL_{50} de esas soluciones a las 24, 72 y 144 h después del tratamiento. Para *B. bassiana*, el detergente disminuyó significativamente la LC_{50} de ese producto solo después del tratamiento de 72 h. Las soluciones fúngicas con detergente no afectaron la germinación conidial de los hongos entomopatógenos. Nuestros resultados encontraron que al mezclar las formulaciones de *B. bassiana*, *M. anisopliae* o clorpirifos con TS-2035 a 0,001% v/v, no controló *P. viburni* mediante la eliminación de su cera epicuticular, pero contribuyó significativamente a la mortalidad.

Palabras Clave: *Beauveria bassiana*; integumento de insectos; manejo integrado de plagas; *Metarhizium anisopliae*; toxicología

In Chile, the obscure mealybug, *Pseudococcus viburni* (Signoret) (Hemiptera: Pseudococcidae), is an economically important fruit pest that causes rejection of cargo when detected on infested produce scheduled for export (Curkovic et al. 2015). This pest species is difficult to adequately control in the field and requires continuous year-round insecticide application. The epidermis of this mealybug characteristically secretes abundant white wax on the dorsal surface of females that prevents penetration of insecticides (Toro et al. 2003) and pathogens (Vincent & Wegst 2004; Pedrini et al. 2007).

Integrated pest management efforts for agriculturally important insect pests endeavor to implement effective and environmentally friendly control methods. One control method that has been successful is the use of synthetic detergents. Small, soft bodied arthropods such as aphids, mealybugs, mites, and whiteflies have been reported to be sensitive to synthetic detergents (Cloyd 2006; Cranshaw 2006; Santibáñez 2010). Detergents, as insecticides, are advantageous because they often have multi-site modes of action, produce relatively little impact on the environment, are low cost, pose fewer legal restrictions on use, and have no residual insecticidal action (Curkovic et al. 2007, 2015; Pavela 2007). In addition to possessing insecticidal properties of their own, some synthetic detergents have been added to pesticides as co-adjuvants to enhance toxicity to control agriculturally important mealybug and mite species because of their surfactant and degreasing properties (Cowles et al. 2000; Curkovic et al. 2007; Sazo et al. 2008).

The insecticidal effect of detergents on insect pests depends on concentration, and is attributed to removal of epicuticle waxes increasing susceptibility to drowning and pathogens (through indirect exposure), dislodgement, etc. (Curkovic & Araya 2004). The objective of our study was to evaluate the effectiveness of an agricultural detergent (TS-2035®) at a non-lethal concentration alone, and as a co-adjuvant

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to control *P. viburni* females when added to formulations containing either *Beauveria bassiana* Vuillemin (Cordycipitaceae), *Metarhizium anisopliae* Sorokin (Clavicipitaceae), or chlorpyrifos.

Materials and Methods

INSECTS

Pseudococcus viburni, previously collected in 2012 from a pomegranate (*Punica granatum* L.; Lythraceae) field in Huechún (33.082900°S, 70.669569°W), central Chile, were used in this study. Mealybugs were reared on potato sprouts grown in the dark at the Insect Behavior and Chemical Ecology Laboratory, Department of Crop Protection, College of Agronomic Sciences, University of Chile, La Pintana, Santiago, Chile. Individuals were maintained in 32 × 21 × 14 cm transparent plastic boxes with side and top screened windows to allow ventilation. Boxes were transferred to a plant growth chamber (model JSPC-420C, JSR Research Inc., Chungehungnan-Do, Korea) at 19.5 °C, about 40% RH, and 16:8 h (L:D) photoperiod (Carpio 2013). Females (1–7 d old) were used in all evaluations. Before exposure to treatments, mealybugs were gently removed from their rearing substrate using a number 2 camel hair artist's brush (M. Grumbacher, Leeds, Massachusetts, USA).

INSECTICIDES AND CHEMICALS

The detergent used, TS-2035® (PACE International, Santiago, Chile), is a neutral (pH 7.0) liquid agricultural detergent that contains anionic (34%) and non-ionic (4%) surfactants, inactive carriers, and water (62%). Two entomopathogenic fungi, *Beauveria bassiana* (strain GHA, 11.3% v/v; Mycotrol ES®, Bioamérica, Santiago, Chile) and 6 mo old *Metarhizium anisopliae* var. *anisopliae* conida (strain Qu-M984 + Tween 80 at 0.05% v/v following provider recommendation) from the Institute of Agricultural Research of Chile, Quilamapu, Chile, were used in the study. The maximum viable fungal concentrations (100% in Figs. 2, 3) were 1.7×10^6 (Mycotrol ES®) and 3.7×10^7 (*M. anisopliae*) colony forming units per mL. All treatments were compared with Lorsban 4E® (chlorpyrifos 48% AI) (Dow AgroSciences Chile S.A., Santiago, Chile) as a standard, applied at 1.2 mL per L (576 ppm) as recommended by the manufacturer.

BIOASSAYS

Two mL aliquots of each treatment were applied to glass Petri dishes (90 × 14 mm) containing *P. viburni* females (20 individuals per dish; n = 5 dishes) using a ST-4 Potter spray tower (Burkard Manufacturing Co. Ltd., Hertfordshire, England) at 15 psi. Afterwards, mealybugs were moved to a clean dish with oleander leaves (*Nerium oleander* L.; Apocynaceae) and placed in a climatic chamber as described earlier. Mortality was recorded at 24, 72, and 144 h post treatment (the latter only for *M. anisopliae*), and determined by the absence of motility upon being slightly prodded with a camel hair artist's brush. Initially, 5 TS-2035 serial concentrations, starting at 1% (v/v), were used to determine a non-lethal concentration (i.e., the greatest concentration not significantly different from the control) for *P. viburni* females. This concentration was then added to the fungal and insecticide formulations mentioned earlier for later bioassays. Controls consisted of distilled water only. The pH values did not statistically differ between the control (7.0 ± 0.1) and the final non-lethal concentration (7.1 ± 0.2).

For fungal formulations, 20 treated *P. viburni* females were placed inside sanitized plastic humidity chambers (10 cm basal diam) with a circle of absorbent paper at the bottom 7 d after exposure. Individuals were then sprayed with 5 mL distilled water and transferred to a climatic chamber with the same temperature and moisture conditions mentioned earlier. Treated insects were checked daily for fungal growth for 1 wk (Fig. 4).

Quantification of epicuticle wax content (i.e., removal) from treated *P. viburni* females was conducted in the Chemistry Laboratory, Department of Agroindustry and Enology, College of Agronomic Sciences, University of Chile (Skoog et al. 2005). The non-lethal concentration of detergent (TS-2035) was applied to 20 individuals using a Potter spray tower. For comparison purposes, the same number of females were sprayed with TS-2035 at 1% v/v and distilled water without detergent as a control treatment. After application, mealybugs were dried at room temperature, then weighed on a BRB32 (Boeco, Germany) analytical scale, transferred to Eppendorf tubes (1.5 mL), and frozen at −20 °C until analysis, following the methods of Buckner et al. (1999). Any remaining wax from individuals was extracted later by immersion in 1 mL chloroform for 1 min. The supernatant was collected with a micropipette, then submitted for UV/VIS spectrophotometer measurements in a PharmaSpec UV-1700 (Shimadzu, Japan), set at 245 nm (Nelson & Charlet 2003). These results allowed quantitative estimates (% w/w) of remaining waxes from treated specimens using an absorbance curve previously described by Santibáñez (2010).

DETERMINATION OF CONIDIAL GERMINATION AND MYCELIAL GROWTH

To verify that germination of fungal formulations was not adversely affected by the addition of TS-2035, each mealybug was exposed to 1× and 10× the non-lethal concentration, as well as no detergent. The latter treatment was used as a control. Four replicates of each fungal product were evaluated by homogeneously sowing 100 µL of 10^6 colony forming units per mL (verified in a Neubauer chamber) in distilled water in Petri dishes with potato dextrose agar at pH 6.0. Dishes were kept in a FOC225E chamber (Velp, Italy) at 20 °C; colony forming units were quantified every 12 h over 7 d. Maximum germination for *B. bassiana* and *M. anisopliae* occurred at 36 h and 144 h, respectively. Germination percent was determined by randomly examining 100 conidia under an Axiostar plus magnifier (Carl Zeiss, Gottingen, Germany) using a contrast phase microscope at 400×. Conidia were considered germinated when a germination tube was present with a length equal or superior to its diam.

STATISTICAL ANALYSIS

All bioassays followed a completely randomized block design with 4 replicates. The blocking criterion was the d replicates were evaluated. Each block included 1 replicate of each treatment applied on the same d. The mean lethal concentration (LC_{50}) for entomopatogenic fungi, detergent, and chlorpyrifos alone and in detergent mixtures, were estimated by Probit analysis as described by Rustom et al. (1989). When mortality occurred in controls, percentages were corrected using the formula: 100 × [(IDT – IDC) / (Ti – IDC)] adapted from Rustom et al. (1989), where IDT = individuals dead by effect of the treatment, IDC = individuals dead in the control, and Ti = total individuals per replicate. The LC_{50} values were calculated within blocks, then compared using the Friedman nonparametric test (for Mycotrol and chlorpyrifos) and the post hoc Tukey HSD (for *M. anisopliae*) (Zar 1996).

Pseudococcus viburni mean percent data were transformed prior to analysis using arc sine, then subjected to tests to verify normality and homocedasticity, respectively (Kuehl 1994). A 2-way ANOVA was then performed on the data, and the Tukey HSD test used to contrast the effects of concentration within each block. If the results did not comply with the above 2 statistical assumptions, then Friedman's test was used. The results of mean pH values of solutions, spore germination, and wax remaining (% w/w) from mealybugs treated with TS-2035 were subjected to a 1-way ANOVA and means compared with Tukey HSD tests (Zar 1996). Differences in all analyses were considered significant at $P < 0.05$, and tests were performed with statistical software SSPS (version 15.0, SPSS Inc., Chicago, Illinois, USA).

Results

At 24 and 72 h after exposure, mealybug mortality increased as TS-2035 concentration increased (Fig. 1). Exposure to detergent concentrations ≥ 0.001% (v/v) turned female *P. viburni* from white to orange as their waxy layer was evidently removed by the treatment. The wax remaining in *P. viburni* females after exposure to 0.001% detergent did not vary significantly with the control (1.2% ± 0.1 vs. 1.2% ± 0.1, respectively), but it did when 1% TS-2035 was used (0.9% ± 0.1). Maximum mortality caused by the detergent was achieved at 24 h at the highest concentration (1%) and was significantly greater than the rest of the concentrations (Fig. 1). However, at 72 h, no significant differences occurred when mortality at 0.1 and 1% were compared. No significant differences in mealybug mortality occurred between 0.01 and 0.1% at either evaluation intervals. No mortality occurred in controls at 24 h and reached less than 10% at 72 h. At both time periods, mealybug mortality to TS-2035 applied at 0.001% did not differ significantly from controls. Thus, this concentration was adopted as the non-lethal concentration for the later fungal and chemical mixture assays.

Mealybug mortality to fungal formulations, with and without TS-2035, increased with concentration at each time period and was significantly greater than controls (Figs. 2, 3). The addition of TS-2035 at 0.001% to either fungal formulation significantly decreased the LC_{50} (about 1 order of magnitude) at each evaluation period with the exception of *B. bassiana*, where there was no difference at 24 h (Table 1). At 24 and 72 h, *P. viburni* mortality to the *B. bassiana* formulation (alone) was significantly greater along with the concentration (Fig. 2A), and a similar trend was observed for the *B. bassiana* + TS-2035 (Fig. 2B).

At 24 h, the *M. anisopliae* formulation alone produced about 40% mealybug mortality at the highest concentration, then increased to slightly above 50% at 72 and 144 h post treatment (Fig. 3A). The addition of TS-2035 continued this trend, and provided about 10 to 30% greater mortality compared with the fungal pathogen alone, at the 2 highest concentrations during all evaluation periods (Fig. 3B). The highest level of *P. viburni* mortality (about 80%) occurred at the greatest concentration of the mixture *M. anisopliae* plus the detergent at 0.001% v/v.

The addition of 0.001% TS-2035 to the *B. bassiana* formulation did not significantly decrease germination of its conidia (Table 2), whereas 10× this concentration significantly reduced germination compared with controls. Unlike *B. bassiana*, the germination of *M. anisopliae* conidia was not significantly affected at any concentration. After 24 h of exposure, typical filamentous white mycelium with synemma-like projections of *B. bassiana* (Fig. 4A–C) were observed emerging from mealybug treated bodies, completely colonizing them in 7 d. However, only 5% of the *M. anisopliae* treated insects exhibited signs of emerging dark-green mycelium and conidia after 7 d (Figs. 4D–F).

Mortality of *P. viburni* exposed to chlorpyrifos with and without TS-2035 also increased with concentration at each time period, with the highest concentration providing the significantly greatest mortality (Fig. 5). It is worth noting also that chlorpyrifos alone at this concentration provided up to 80% mortality of mealybugs that increased to about 93% with the addition of the detergent (Table 3). Generally, the addition of TS-2035 (0.001% v/v) to chlorpyrifos significantly decreased the LC_{50} at both evaluation periods compared with the insecticide alone.

Discussion

Several authors have reported that it is very important to use co-adjuvants to improve the pathogenicity of entomopatogenic fungi (Holder 2005; Jin et al. 2008; Martínez 2010; Mishra et al. 2013). In our study, we found that adding the detergent TS-2035 at a non-lethal concentration of 0.001% v/v to *B. bassiana* and *M. anisopliae*, the mortality of female *P. viburni* was increased significantly. However, not all detergents are adequate as fungal co-adjuvants. A large part of their effectiveness depends on the suspension pH. For *B. bassiana*, conidial adherence to hydrophobic surfaces (like the epicuticle wax of *P. viburni*) is optimal at a pH range of 7.0 to 8.0 (Holder 2005), and its proteolytic enzyme activity at 6.5 to 8.0 (Raja et al. 2010). Our results indicate no changes in pH values that were observed when the detergent was added to this fungal formulation at 0.001% v/v, keeping it close to neu-

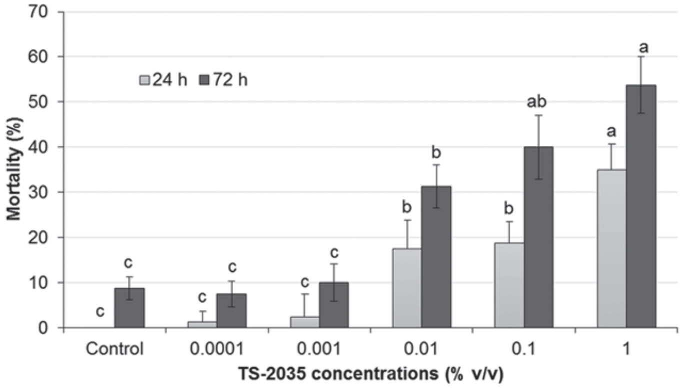

Fig. 1. Mortality (%) of *Pseudococcus viburni* females after exposure to several concentrations of TS-2035.

Table 1. LC_{50} of *Pseudococcus viburni* females exposed to *Beauveria bassiana* and *Metarhizium anisopliae* with and without TS-2035[a].

Treatments	Evaluation time (h)	LC_{50} (CFU per mL)[b]
B. bassiana	24	2.6×10^4 c
B. bassiana + NLC[a]	24	4.1×10^4 c
B. bassiana	72	1.6×10^4 b
B. bassiana + NLC	72	9.5×10^3 a
M. anisopliae	24	8.6×10^7 c
M. anisopliae + NLC	24	8.8×10^6 ab
M. anisopliae	72	3.3×10^7 c
M. anisopliae + NLC	72	7.8×10^6 a
M. anisopliae	144	3.0×10^7 bc
M. anisopliae + NLC	144	6.1×10^6 a

[a]at 0.001% v/v (non-lethal concentration).
[b]CFU = colony forming units. The LC_{50} values with different letters, within each entomopathogenic fungus, are significantly different. Friedman's non-parametric test was used in the tests with *B. bassiana* and Tukey HSD test was used for *M. anisopliae* ($P < 0.05$).

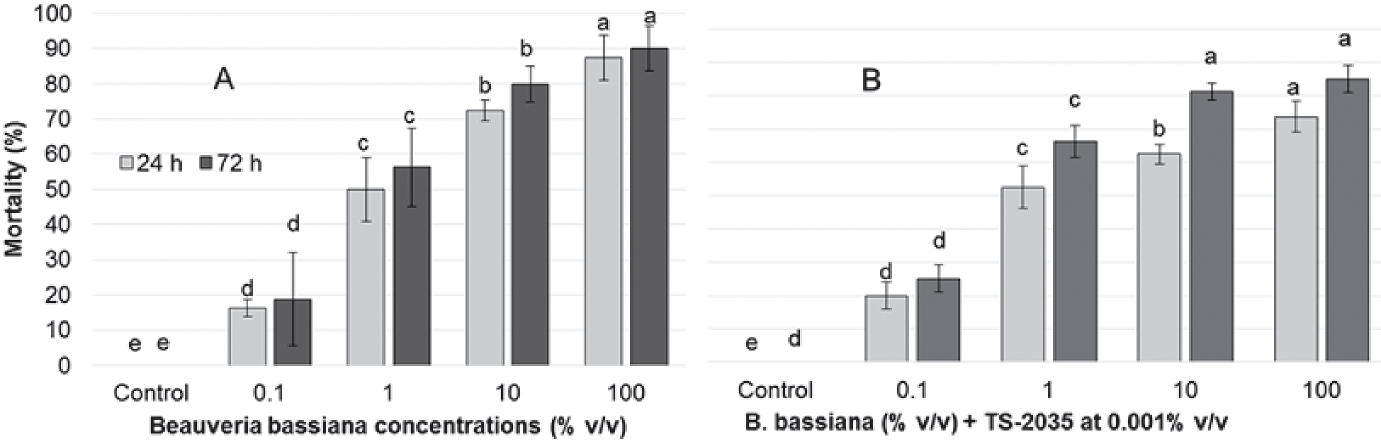

Fig. 2. Mortality (%) of *Pseudococcus viburni* females to (A) *Beauveria bassiana* alone, and (B) mixed with a nonlethal concentration of TS-2035.

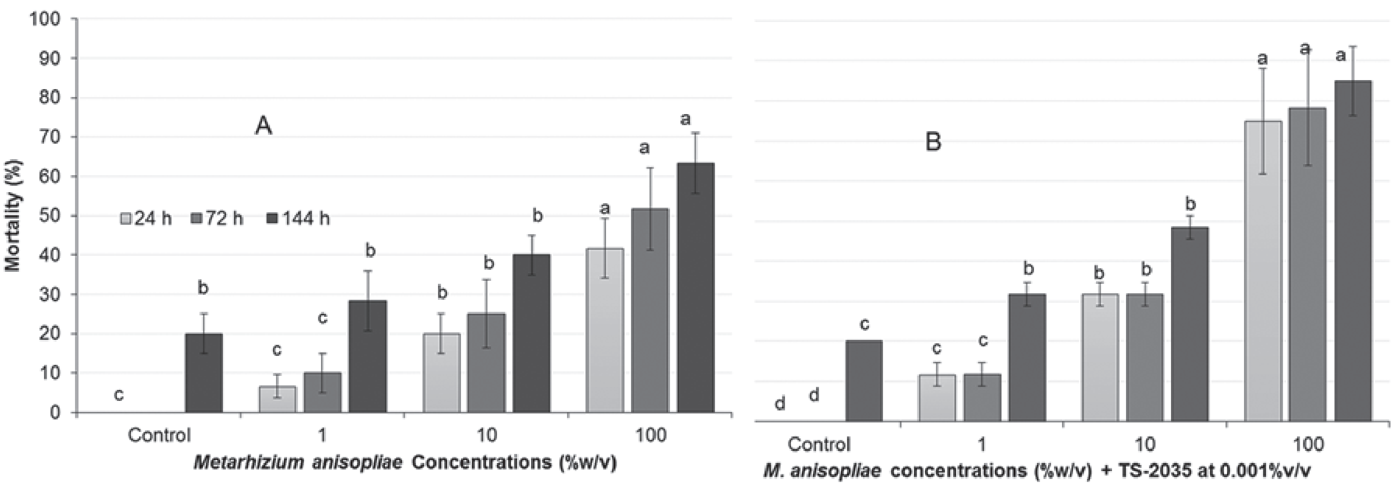

Fig. 3. Mortality (%) of *Pseudococcus viburni* females to (A) *Metarhizium anisopliae* alone, and (B) mixed with a nonlethal concentration of TS-2035.

tral. However, Tamerler et al. (1998) indicate that *M. anisopliae* needs a pH of 5.7 to 6.2 for production of its secondary metabolites and optimum development, a bit lower than the levels we measured for this formulation.

Several authors have reported that some anionic surfactants, like those present in TS-2035 (Santibáñez 2010), had a delaying (Santos et al. 2011) or negative effect (Holder 2005; Jin et al. 2008; Mishra et al. 2013) on conidial germination of *B. bassiana* and *M. anisopliae*, as well as other cationic and non-ionic surfactants at concentrations greater (0.1%) than those used in our study (Holder 2005). This is possibly due to differences in fungal cell membranes that affect permeability and cause the loss of amino acids (Luz & Batagin 2005; Mishra et al. 2013), or accumulation of substances around them that prevent germination (Mishra et al. 2013). In our study, fungal mixtures with 0.001% TS-2035 did not affect the conidial germination, but it did adversely affect germination above that concentration. Another effect of detergents is fast drying, which is frequently observed in small or soft-body insects (Po-

Table 2. Mean percent conidial germination (± SE) of *Beauveria bassiana* (72 h), *Metarhizium anisopliae*) (144 h) with and without TS-2035.

	Conidial germination (%)[a]	
TS-2035 (% v/v)	*Beauveria bassiana*	*Metarhizium anisopliae*
0.000	52 ± 3 a	37 ± 8 a
0.001	44 ± 8 a	35 ± 9 a
0.010	37 ± 7 b	30 ± 5 a

[a]Mean conidial germination values in columns, for each fungal species, with different letters are significantly different, Tukey HSD test ($P < 0.05$).

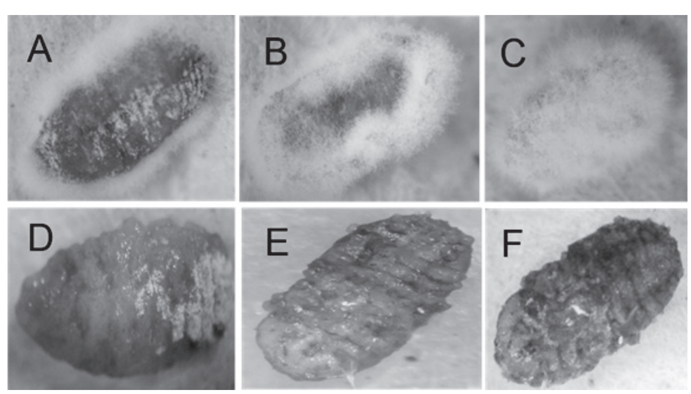

Fig. 4. Mycelium growth of (A-C) *Beauveria bassiana*, and (D-F) *Metarhizium anisopliae* on *Pseudococcus viburni* females at 24, 72, and 172 h after exposure.

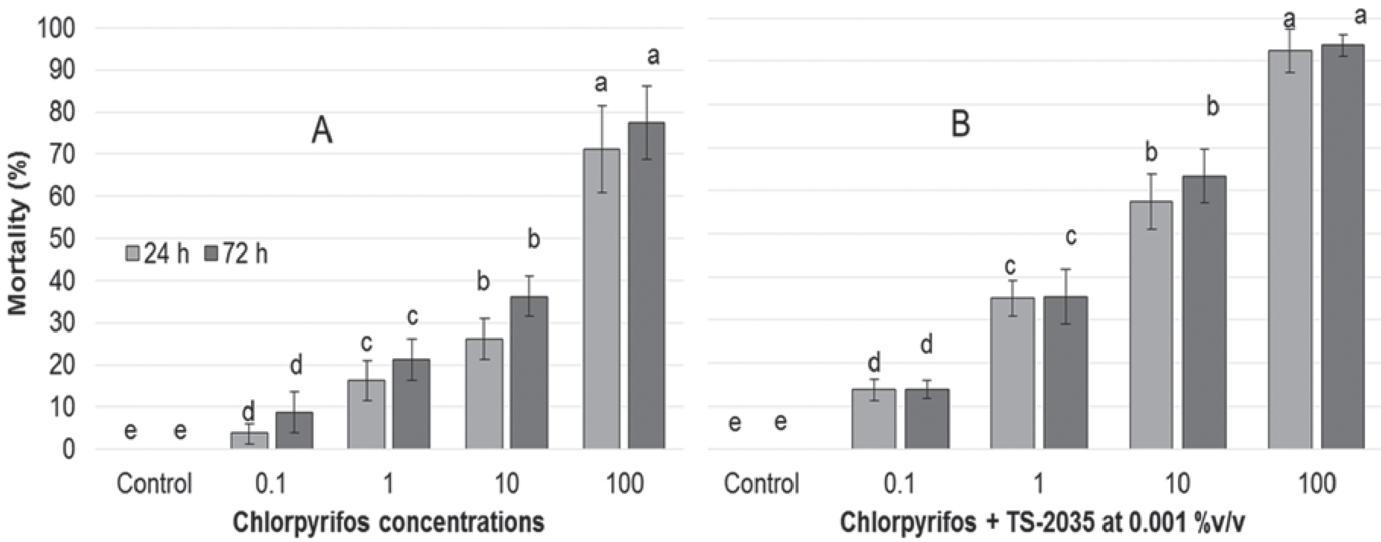

Fig. 5. Mortality (%) of *Pseudococcus viburni* females to (A) chlorpyrifos alone, and (B) mixed with a nonlethal concentration of TS-2035.

prawski et al. 1999; Chirinos et al. 2007) like *P. viburni* females after treatment. Rapid drying was observed by Santibáñez (2010) and may explain why (in our experiments) we were unable to support mycelium growth or sporulation in some of the dead insects. We observed that wax removal from mealybugs treated with the non-lethal concentration of TS-2035 was not different from controls. Santibáñez (2010) similarly found no difference from controls with *P. viburni* adult females after exposure to the same detergent at 3% v/v. This evidence suggests that wax removal (degreasing) did not contribute to the lethal effect from their mixture, nor from ours.

Amnuaykanjanasin et al. (2013) indicated that strain BCC2660 of *B. bassiana* (different than the one in our study) required 6 to 7 d to cause complete mortality on the cassava mealybug *Phenacoccus manihoti* Matile-Ferrero (Hemiptera: Pseudococcidae) when sprayed at a 10^8 conidia per mL, greater than what we used herein. Spraying *P. viburni* females with the same strain of *M. anisopliae* used in our study, Pereira et al. (2011) reported a LC_{50} of 3.2×10^4 conidia per mL with a mean lethal time (LT_{50}) of 7.4 d on females (closer to our results) but at a lower application rate. Furthermore, Pereira et al. (2011) found that the *M. anisopliae* strain we tested reached a LT_{50} on *P. viburni* in 7.7 to 10.0 d at 7.3×10^5 to 4.9×10^9 conidia per mL, requiring longer time than our study. Not surprisingly, we also found that chlorpyrifos was acutely more toxic than the fungal formulations to control female *P. viburni.* However, when this insecticide was mixed with TS-2035 at 0.001% v/v, toxicity increased by almost 9.4× (24 h after exposure) and about 5.6× at 72 h compared with chlorpyrifos alone. Based on our results it would be valuable to evaluate, under field conditions, the additional toxic effects of adding various detergents (at non-lethal concentrations) as co-adjuvants to a variety of insecticides on the mortality of *P. viburni* and other mealybug pests.

Table 3. LC_{50} of *Pseudococcus viburni* females exposed to chlorpyrifos with and without TS-2035 (0.001% v/v) at 24 and 72 h.

Treatments	Evaluation times (h)	LC_{50}[a] (ppm)
Chlorpyrifos only	24	170 c
Chlorpyrifos + TS-2035	24	18 a
Chlorpyrifos only	72	85 b
Chlorpyrifos + TS-2035	72	15 a

[a]LC_{50} values with different letters are significantly different, Tukey HSD test ($P < 0.05$).

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