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Tetranychus urticae (ACARI: TETRANYCHIDAE) EN FRUTILLAS: INTERACCIÓN DE DOS AGENTES DE CONTROL BIOLÓGICO, HONGOS ENTOMOPATÓGENOS Y ÁCAROS DEPREDADORES

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Tetranychus urticae **(ACARI: TETRANYCHIDAE) EN FRUTILLAS: INTERACCIÓN DE DOS AGENTES DE CONTROL BIOLÓGICO, HONGOS ENTOMOPATÓGENOS Y ÁCAROS DEPREDADORES**

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Colegio de Postgraduados, 2019

RESUMEN

Tetranychus urticae Koch es una plaga de distribución mundial y afecta a diversos cultivos. El principal método de manejo es mediante pesticidas químicos. Sin embargo, el desarrollo de resistencia ha hecho que se busquen otras alternativas de control. Bajo condiciones de laboratorio, se evaluó la eficacia de los ácaros depredadores *Phytoseiulus persimilis* Athias-Henriot (especialista) y *Neoseiulus californicus* McGregor (generalista), solos y en combinación con hongos entomopatógenos para reducir poblaciones de *T. urticae*. Primero se evaluó la susceptibilidad relativa de los ácaros depredadores y *T. urticae* hacia la infección por aislamientos de los hongos *Metarhizium anisopliae* (Metchnikoff) Sorokin, *Beauveria bassiana* Bals. Vuill, *Lecanicillium lecanii* R. Zare & W. Gamsy e *Isaria fumosorosea* Wize. En general, *T. urticae* fue la especie más susceptible en comparación con los ácaros depredadores. Los aislamientos de *B. bassiana* y *M. anisopliae* causaron las mortalidades más altas. Cuando se evaluó la mortalidad de *T. urticae* expuestos a cada depredador, solos y en combinación con hongos, la mayor mortalidad de adultos y ninfas de *T. urticae* se encontraron cuando ambos agentes de control biológico fueron usados en combinación, sin diferencias entre especies de depredadores, pero con un mayor efecto

de los aislamientos de *B. bassiana* y *M. anisopliae*. Los resultados muestran que la combinación de hongos y ácaros depredadores tiene potencial para el control biológico de *T. urticae*.

Palabras clave: Ácaros, Frutillas, Vectores, Patogenicidad, Control Biológico.

Tetranychus urticae **(ACARI: TETRANYCHIDAE) IN BERRIES: INTERACTION OF TWO AGENTS OF BIOLOGICAL CONTROL, ENTOMOPATOGEN FUNGI, AND PREDATOR MITES**

Oscar Daniel Castillo Ramírez, M. en C.

Colegio de Postgraduados, 2019

ABSTRACT

The mite, *Tetranychus urticae* Koch, has a worldwide distribution and is a pest on several agricultural crops. Chemical pesticides have been the main control strategy. However, resistance has encouraged the development of more effective and sustainable control strategies. Under laboratory conditions, we evaluated the control efficacy of the predatory mites, *Phytoseiulus persimilis* Athias-Henriot (specialist) and *Neoseiulus californicus* McGregor (generalist), alone or as vectors of entomopathogenic fungi (i.e. in combination with fungi). First, we evaluated the relative susceptibility of the predatory mites and *T. urticae* to infection by *Metarhizium anisopliae* (Metchnikoff) Sorokin, *Beauveria bassiana* Bals. Vuill, *Lecanicillium lecanii* R. Zare & W. Gams and *Isaria fumosorosea* Wize. Overall, *T. urticae* was more susceptible to fungi than the predatory mites; *B. bassiana* and *M. anisopliae* isolates caused greatest mortality. We then quantified *T. urticae* mortality due to one or other of the two predatory mite species alone or when contaminated with conidia of each of the four fungal species. Greatest *T. urticae* mortality was achieved when predatory mites were used in combination with entomopathogenic fungi; there was no difference between the two predatory mites. The greatest mortality in adults and nymphs, and the lowest number of eggs laid by *T. urticae* was achieved where predatory mites were contaminated with,

and vectoring, conidia of *B. bassiana* and *M. anisopliae*. Combining predatory mites and entomopathogenic fungi has potential for biological of *T. urticae*.

Key words: Mites, Strawberries, Vectors, Pathogenicity, Biological Control.

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CONTENIDO

LISTA DE FIGURAS

Página

LISTA DE CUADROS

Página

INTRODUCCIÓN GENERAL

Al igual que otros vegetales cultivados bajo cubierta, las frutillas son infestadas por un amplio rango de plagas chupadoras (Dara, 2017), cuya importancia económica es muy alta, debido a que la mayoría de ellas son vectores de fitopatógenos, causantes de numerosas enfermedades (Booth *et al.,* 2007). Entre estos artrópodos plaga, se ubican los ácaros fitófagos. En general, existen diversas familias de ácaros de importancia agrícola, por su alto grado de daño por fitofagia, transmisión de diversos patógenos (Eriophyidae), así como, los desórdenes fisiológicos que ocasionan a un amplio rango de plantas cultivadas, entre ellas las berries (Childers *et al.,* 1996; Chandler, 2010). Especies de Tenuipalpidae (Brannen y Horton, 2013; Akyazi *et al.,* 2017) afectan el desarrollo normal de sus plantas hospedantes. Mientras que, miembros de la familia Tarsonemidae, succionan la savia de brotes tiernos, hojas y frutos, (Tuovinen y Lindqvist, 2010; Labanowska *et al.,* 2015). Sin embargo, las especies de la familia Tetranychidae son consideradas una de las plagas fitófagas más devastadoras a nivel mundial (Van Leeuwen *et al.,* 2010; Livinali *et al.,* 2014; El-Ela, 2014).

La principal táctica de control empleada mundialmente para reducir las infestaciones de ácaros fitófagos en la agricultura bajo protección es el combate químico (Ilias *et al.*, 2014). Sin embargo, la constante presión de selección que se ejerce sobre estos artrópodos promueve el desarrollo de resistencia a estos insumos, principalmente, al producto comercial más abundante en el mercado (abamectina) (Çağatay *et al.,* 2018). En las últimas décadas, se han documentado 417 casos de resistencia a 93 diferentes moléculas presentes en los plaguicidas químicos (Van Leeuwen *et al.,* 2015). Aunado a los casos de resistencia, el abuso de sustancias químicas para el control de

ácaros fitófagos ha desarrollado problemas sociales y ecológicos a gran escala en México y en el resto del mundo (García *et al.,* 2018).

Los ácaros fitófagos poseen diversos enemigos naturales, de los cuales los hongos entomopatógenos son excelentes agentes de control, incluso algunas de estas especies se encuentran asociadas exclusivamente a ácaros como: *Hirsutella thompsonii* y *Neozygites floridana* (Chandler *et al.,* 2010). Diversas investigaciones han demostrado la efectividad de: *Beauveria bassiana*, *Metarhizium anisopliae*, *Isaria fumosorosea* y *Lecanicillium lecanii* para reducir poblaciones de ácaros, ejemplo: *Tetranychus urticae* (Drummond y Groden, 2000; Shah y Pell, 2003; Booth *et al.,* 2007; Dara, 2017; Dogan *et al.,* 2017). Así mismo, se ha documentado la capacidad de miembros de la familia Phytoseiidae como reguladores biológicos de poblaciones de ácaros fitófagos (Rosenheim, 1998). Bajo este contexto, el empleo de *Phytoseiulus persimilis* y *Neoseiulus californicus* son considerados excelentes agentes de control capaces de suprimir poblaciones de ácaros fitófagos incluyendo *T. urticae* (Greco *et al.,* 2011; Charles *et al.,* 2012; Kazak *et al.,* 2015).

Entre otros factores, la efectividad de los hongos entomopatógenos para reducir poblaciones de ácaros fitófagos se ve limitada por su método de aplicación (Bateman *et al.*, 2007), en contra parte, se ha documentado que esta efectividad se ve incrementada cuando se propicia un mayor y mejor contacto de la unidad infectiva (espora) con su organismo hospedante (ácaro fitófago), ya sea por medio de mecanismos propios de cada especie de entomopatógeno o por medio del arrastre de otros artrópodos, presentes o introducidos, actuando como los vectores de estos agentes infecciosos (Roy y Pell, 2000; Vega *et al.,* 2009; Lin *et al.,* 2017). La posibilidad de emplear hongos entomopatógenos y ácaros depredadores en combinación, para el control biológico de ácaros fitófagos ha sido previamente sugerida. A pesar de que existen reportes sobre

los efectos negativos en la fisiología y comportamiento que sufren los ácaros depredadores, cuando son expuestos a especies de hongos entomopatógenos (Ullah y Lim, 2017; Wu *et al.*, 2018), se ha demostrado que éstos son de bajo riesgo proporcionando una ventaja para su uso combinado.

Esta investigación tiene por objetivo, estudiar la susceptibilidad de *T. urticae*, la especie fitófaga más importante, y la de dos ácaros depredadores *Phytoseiulus persimilis* y *Neoseiulus californicus* a la infección por hongos entomopatógenos, y su posible uso combinado para reducir poblaciones de *T. urticae*.

Objetivo e hipótesis

Objetivo general:

Estudiar la susceptibilidad de *T. urticae* y la de dos ácaros depredadores, a cuatro especies de hongos entomopatógenos y su posible empleo combinado para reducir poblaciones de *T. urticae*.

Objetivos específicos:

- Determinar la susceptibilidad de *Tetranychus urticae, Phytoseiulus persimilis y Neoseiulus californicus* a la infección por los hongos entomopatógenos.
- Determinar la interacción de ácaros depredadores y hongos entomopatógenos en la reducción de poblaciones de *T. urticae*

Hipótesis:

La combinación de ácaros depredadores y hongos entomopatógenos reducirá más eficientemente poblaciones de *T. urticae*, comparado con ambos agentes de control biológico de manera individual.

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CHAPTER I: INTERACTION BETWEEN PREDATORY MITES (ACARI: PHYTOSEIIDAE) AND ENTOMOPATHOGENIC FUNGI IN *Tetranychus urticae* POPULATIONS (ACARI: TETRANYCHIDAE)[1]

ABSTRACT

The mite, *Tetranychus urticae* Koch, has a worldwide distribution and is a pest on several agricultural crops. Chemical pesticides have been the main control strategy. However, resistance has encouraged the development of more effective and sustainable control strategies. Under laboratory conditions, we evaluated the control efficacy of the predatory mites, *Phytoseiulus persimilis* Athias-Henriot (specialist) and *Neoseiulus californicus* McGregor (generalist), alone or as vectors of entomopathogenic fungi (i.e. in combination with fungi). First, we evaluated the relative susceptibility of the predatory mites and *T. urticae* to infection by *Metarhizium anisopliae* (Metchnikoff) Sorokin, *Beauveria bassiana* Bals. Vuill, *Lecanicillium lecanii* R. Zare & W. Gams and *Isaria fumosorosea* Wize. Overall, *T. urticae* was more susceptible to fungi than the predatory mites; *B. bassiana* and *M. anisopliae* isolates caused greatest mortality. We then quantified *T. urticae* mortality due to one or other of the two predatory mite species alone or when contaminated with conidia of each of the four fungal species. Greatest *T. urticae* mortality was achieved when predatory mites were used in combination with entomopathogenic fungi; there was no difference between the two predatory mites. The greatest mortality in adults and nymphs, and the lowest number of eggs laid by *T. urticae* was achieved where predatory mites were contaminated with,

[1] Castillo-Ramírez, O.D., Guzmán-Franco, A.W., Santillán-Galicia, M.T., Tamayo-Mejía, F. 2019. Interaction between predatory mites (Acari: Phytoseiidae) and entomopathogenic fungi in *Tetranychus urticae* populations (Acari: Tetranychidae). Journal of Pest Science (**Submitted**).

and vectoring, conidia of *B. bassiana* and *M. anisopliae*. Between 3 and 13% of predatory mites vectoring conidia became infected. Combining predatory mites and entomopathogenic fungi has potential for biological of *T. urticae*.

Keywords: Spider mites, biological control, *Phytoseiulus persimilis*, *Neoseiulus californicus, B. bassiana, M. anisopliae, Isaria fumosorosea, Lecanicillium lecanii*

Key message

- *Tetranychus urticae* can be controlled using predatory mites (*Phytoseiulus persimilis*/ *Neoseiulus californicus*), or entomopathogenic fungi; few studies have considered using both natural enemy groups in combination
- *T. urticae* was more susceptible to fungal infection than predatory mites when fungi were applied directly as a suspension. *Beauveria bassiana* and *Metarhizium anisopliae* caused greater mortality than *Isaria fumosorosea* and *Lecanicillium lecanii*
- Predatory mites vectoring fungal conidia caused greater *T. urticae* mortality than predatory mites alone. Predatory mites vectoring *B. bassiana* and *M. anisopliae* conidia caused greater mortality than predatory mites vectoring the other two isolates. No difference was observed between the predatory mite species.
- Mortality of predatory mites vectoring conidia was very low (3-13%), suggesting the combined use of fungi and predatory mites for control of *T. urticae* is possible. Field experiments are now needed to confirm these results.

1.1 Introduction

Tetranychus urticae Koch is a pest worldwide and affects approximately 1100 plant species (Çağatay et al. 2018). Currently, chemical pesticides are the main control strategy. However, because of their intensive use resistance has developed; to date more than 450 cases of resistance against 92 active ingredients have been reported (Van Leuween et al. 2015). All these factors combined make this mite one of the most important pests of agricultural crops.

Biological control is a very important strategy for reducing *T. urticae* populations (Gerson and Weintraub 2007). Entomopathogenic fungi are amongst the strongest candidates for biological control of *T. urticae* , as reported by several authors (e.g. Chandler et. al. 2000, 2005; Bugeme et al. 2015; Ullah and Lim 2015; Dogan et al. 2017). Traditionally, conidia of entomopathogenic fungi are applied (sprayed) as suspensions. However, conidia do not always reach the target, or at least not in sufficient quantities to cause infection and achieve significant reductions on pest populations. Research suggests that using particular arthropods to 'vector' conidia directly to the pest has great potential as a delivery method. Dromph (2001) reported the dissemination of *Beauveria bassiana* Bals. Vuill. and *Metarhizium anisopliae* Sorokin by springtails. Al-Mazra'Awi et al. (2007) studied dissemination of *B. bassiana* conidia by *Apis mellifera* L. Dissemination of conidia by natural enemies (insects or mites) has greatest potential as the conidia are delivered directly to the shared host (Baverstock et al. 2010; Saito and Brownbridge 2016; Lin et al., 2017). For example, *Amblyseius swirskii* Athias-Henriot, and *Neoseiulus cucumeris* Oudemans successfully vectored *B. bassiana* conidia to *Diaphorina citri* Kuwayama and caused infection under laboratory conditions (Zhang et al. 2015). The combination of *Phytoseiulus persimilis* Athias-Henriot and *Neozygites floridana* (J. Weiser & Muma) Remaud. & S. Keller reduced *T.*

urticae populations significantly compared with conventional release of the fungus alone (Trandem et al. 2016).

Before releasing combinations of natural enemies in the field, it is desirable to evaluate their compatibility with each other (Midthassel et al. 2016). For example, when combining a predatory mite and an entomopathogenic fungus, success is more likely if the predatory mite is less susceptible to the fungus than the target pest (Wu et al. 2016). Some negative effects on predatory mites, including direct infection by the fungus, have been reported (Duso et al. 2008; Wu et al. 2015). For example, *B. bassiana* can affect the oviposition, longevity and fecundity of the predatory mite *P. persimilis* (Duso et al. 2008; Seiedy et al. 2012; Ulla and Lim 2017). However, despite the negative effects caused by entomopathogenic fungi, their combined use with predatory mites may still represent an efficient approach to reduce *T. urticae* populations.

Predatory mites are important natural enemies of *T. urticae* (Barber et al. 2003). The two predatory mite species most commonly used for the biological control are *P. persimilis* and *Neoseiulus californicus* McGregor (Opit et al. 2004; Vergel et al. 2011; Kamel et al. 2018; Fathipour et al. 2018; Choh et al. 2017; Zheng et al. 2017; Moghadasi et al. 2013; Amoah et al. 2016). Specialist mite predators such as *P. persimilis* are effective predators with efficient foraging behaviours (Blümel and Walzer 2002). Generalist species, such as *N. californicus*, can switch hosts in adverse conditions to find alternative food sources (Greco et al. 2011). Behavioral differences between generalist and specialist predators are likely to influence their role as vectors of entomopathogenic fungi. Attributes of the fungi could also influence the ease with which they could be vectored. For example, conidia of *Lecanicillium lecanii* R. Zare & W. Gams are covered in mucilage (Humber 1997), which could mean they attach more efficiently to the vector´s cuticle, and are consequently vectored more efficiently into host populations, compared with conidia of *B.*

bassiana or *M. anisopliae* that have no mucilage. We hypothesise that a specialist predatory mite covered in mucilaginous conidia are likely to cause greater mortality in *T. urticae* populations than generalist predators covered in conidia without mucilage. To test this, we first compared the susceptibility of *T. urticae* and the predatory mites *P. persimilis* and *N. californicus* to isolates from four fungal species: *B. bassiana*, *M. anisopliae*, *Isaria fumosorosea* Wize and *L. lecanii*. We then quantified *T. urticae* mortality due to one or other of the two predatory mite species in the presence or absence of conidia of each of the four fungal species.

1.2 Material and Methods

1.2.1 Tetranychus urticae colony and predatory mites

Adult *T. urticae* were obtained from blackberry crops in 2017 and have been maintained on bean plants (*Phaseolus vulgaris* L.) since then. Two-week old bean plants grown in pots (1 litre) were used to feed the mites and replaced as required. The colony was maintained at 25 °C, 70 % RH and a light: dark regime of 16:8 h. To obtain adults of the same age for experiments, between 40 and 50 adults were transferred to clean bean plants and incubated under the same conditions for 72 h. Adults were then removed and leaflets containing eggs were allowed to develop. After 10 to 12 days, adults were removed from leaflets and used in experiments.

The predatory mites *Neoseiulus californicus* (Spical®) and *Phytoseiulus persimilis* (Spidex®) (Koppert Biological Systems™, Queretaro, Mexico) were used. Thirty adults from each species were placed on bean leaflets, provided with *T. urticae* individuals of mixed ages as food, and incubated under the same conditions as the *T. urticae* colony. After 5 days, adults were

removed leaving only eggs and these were maintained with *T. urticae* individuals until they reached the adult stage, when they were used in experiments.

1.2.2 Entomopathogenic fungi

We used one isolate of each of the species *M. anisopliae* (Ma129), *B. bassiana* (Bb88), *I. fumosorosea* (Pfr4) and *L. lecanii* (ARSEF2009). All isolates were retrieved from -80 °C storage and grown on Sabouraud dextrose agar (SDA) at 25 °C in Petri dishes containing 15 mL of medium for 15 days in complete darkness. Conidia and mycelium were then removed from the dishes and deposited into sterile 50 mL centrifuge tubes containing 25 mL of 0.03% Tween 80. The centrifuge tubes were vortexed for 5 mins. Suspensions were then filtered into a new sterile centrifuge tube through a double layer of sterile cloth to remove mycelium from the resulting conidial suspensions. Conidia concentration was estimated using a haemocytometer and adjusted to a final concentration of 1×10^8 conidia mL^{-1}. Before experiments, conidial viability was determined following the methods described by Inglis et al. (2012); germination rates were always above 95%. For experiments, all conidial suspensions were maintained at 4 °C for a maximum of 12 h prior to use.

1.2.3 Susceptibility of mites to fungal isolates

The susceptibility of *T. urticae*, and the predatory mites *N. californicus* and *P. persimilis* were determined using the same procedures. Arenas for all experiments were prepared as follows: 40 mm diameter bean leaf disks were placed individually into the bases of 40 mm diameter Petri dishes containing 5 mL of 1.5% water-agar. Leaf disks were placed adaxial side uppermost. Using a fine brush, 20 adult mites were placed onto each leaf disk. Replicate leaf disks for each treatment were inoculated together; 5 mL of each conidial suspension was applied using an acrylic cylinder

(30 cm diameter × 50 cm height, with a 45° inclination) fitted with a cone spray nozzle (Spraying Systems, Wheaton, IL, USA), attached to an air compressor at 20 psi. Control treatments were inoculated in the same way but using 5 mL of 0.03% Tween 80 only. Between isolates and control treatments, the nozzle was sterilized by passing 10mL of 3% sodium hypochlorite through it, followed by 10 mL of 70% ethanol and two rinses of 10 mL sterile distilled water. Treated mites were transferred to new, clean arenas using a fine brush. All arenas were incubated at 25 °C, 60 % RH and a light: dark regime of 16:8 h. Mortality was recorded every 24 h for 6 days. Dead mites were transferred to 40mm diameter Petri dishes containing 5 mL of 1.5 % water-agar and sporulation recorded as an indication of death due to infection. The susceptibility of each mite species to the four fungal isolates were evaluated separately. For each mite species, there were three replicate arenas per treatment and control and the complete experiment was repeated on four separate occasions.

For statistical analysis, residual maximum likelihood (REML) meta-analysis was used. This set of analyses was used to combine the experiments done for each mite species and compare treatments (isolates) in common (Payne et al. 2005). First, the error model was estimated for each experiment (mite species) using Linear Mixed Models. Secondly, using REML meta-analysis, the effect of all treatments was compared using a contrast structure where, as a fixed model, the mortality of each mite species was compared, followed by a comparison amongst each fungal isolate and their interaction assessed. All comparisons were made considering the within-experiment error estimated by the linear mixed model for each experiment (mite species) as the random model for the analysis. Mortality of control treatments were excluded from analysis, as they never exceeded 10% and none sporulated, which confirmed that no cross contamination had occurred. All analyses were done using GenStat v.8 (Payne et al. 2005).

1.2.4 Transmission of entomopathogenic fungi by predators

For this experiment, the ability of each predatory mite to transmit (vector) each of the four fungal isolates to *T. urticae* populations was evaluated; each predatory mite species was evaluated separately. For each mite predator and fungal isolate combination there were three replicates and the complete experiment was repeated on four occasions. For each combination, three treatments were tested: 1) 25 *T. urticae* adults, 2) 25 *T. urticae* adults and five predators, and 3) 25 *T. urticae* adults and five predators vectoring conidia.

1.2.4.1 Mite predators and fungal conidia

Mite predators were obtained as described previously. For this experiment, and in order to obtain sufficient quantities of conidia, all isolates were produced on sterile rice. For this, 150 g of damp rice were sterilized in polyethylene bags for 20 m at 120 °C and 15 psi. Bags were allowed to cool for 24 h before use. Each bag was inoculated with 15 mL of a 1×10^8 conidia mL^{-1} suspension of one of the isolates and incubated for 2 weeks at 25 °C in complete darkness. Bags were then opened and dried at 25 °C in a drying oven for 1 week and conidia were separated from the rice using a sieve with a 300 µM mesh. The conidia collected were deposited in sterile 50-mL centrifuge tubes and maintained at 5 °C for no longer than three weeks prior to use. A separate production batch was used on each occasion the experiment was done. Before experiments, viability of conidia was tested and germination was always above 95%.

1.2.5 Experimental procedure

Experimental arenas were as follows: 60 mm diameter bean leaf disks were placed, adaxial side upper most, in the base of a 60-mm diameter Petri dishes, each containing cotton moistened with 50 mL of sterile distilled water. Mites (*T. urticae*) were allowed to establish in the arenas for 24 h prior to experimentation. After that, and depending on the treatment, five predatory mites, clean or vectoring conidia, were introduced into each arena. The method for contaminating predatory mites with conidia was the same for all isolates. Specifically, a 2mL Eppendorf tube was modified and two 200-µL Eppendorf tubes were attached to it, one at each end (Fig. 1a. Five hundred mg of conidia and mycelium were deposited into each 2 ml tube (Fig. 1b). Fifteen predatory mites were then placed inside the 2 mL tube and incubated at the same conditions as the colony for 12 hours with no access to food; both open ends of the 200-µL tubes were closed to prevent mites from escaping. After incubation, the device was attached to the arena (Fig. 1c) and the end of the 200 µL tube entering the arena was opened to allow the predatory mites to move into the arena, which already contained adult female *T. urticae*. The arena with the device attached was exposed to a heat source (Fig. 1d) for 10 min to encourage the predatory mites to move down into the arena. The arena was covered with black paper during this time to prevent the temperature within the arena from increasing during exposure to the heat source. After 10 m, the cover of the Petri dish attached to the device was removed and replaced with a clean cover. Of the 15 predatory mites that had entered the arena, ten were immediately removed and placed in 200-µL Eppendorf tubes containing 100 µL of sterile 0.03% Tween 80 solution, and used to estimate the number of conidia that they had become contaminated with. To calculate this, tubes containing the ten predatory mites were first vortexed for 2 m and then centrifuged at 12,000 rpm for 30 s. The number of conidia per mite was determined from the number of conidia per mL in the resulting

suspension, as counted in a Neubauer haemocytometer (Table 1). The experimental arenas were incubated at 25 °C, 60 % RH and a light: dark regime of 16:8 h, and observed every 3 days for 9 days. On each recording occasion, mortality of *T. urticae* adults and nymphs was noted; dead mites (*T. urticae* and predators) were removed and incubated as described previously to confirm whether cause of mortality was due to infection. At the same time, the number of viable *T. urticae* eggs were quantified. Egg viability was determined based on egg morphology; eggs that were turgid, globular and translucent were considered viable while eggs that were flat and dried or with obvious mycelial outgrowth were considered dead. Total mortality (combining the effect of predation and/or fungal infection) was used in analysis.

1.2.6 Statistical analysis

Analyses were done separately for each predatory mite species. For *T. urticae* adult and nymphal mortality data, logistic regression analysis was used, assuming a binomial distribution. The same approach was used for each developmental stage. A hierarchical contrasts structure was used. Firstly, the *T. urticae* treatment (control) only was compared with *T. urticae* plus predatory mite treatments (combining clean predatory mites and those vectoring conidia). Secondly, a comparison of *T. urticae* mortality data was made between treatments where only predatory mites were used versus when predatory mites were vectoring conidia. A final comparison was amongst the treatments with predatory mites vectoring conidia from different fungal isolates. Data on the number of viable eggs amongst treatments were analysed using ANOVA with the same hierarchical contrast structure used previously.

1.3 Results

1.3.1 Susceptibility of mites to fungal infection

There were significant differences in susceptibility to fungal species amongst the three mite species ($F_{2, 4.2}$=72.13, *P*=0.003). Overall, *T. urticae* was the most susceptible to fungal infection (Fig. 2). There were significant differences in the level of infection achieved by each isolate ($F_{3, 122.7}$=178.28, *P*<0.001). The greatest infection rates were caused by *B. bassiana* (Bb88) and *L. lecanii* (ARSEF2009) isolates (Fig. 2). However, there was a significant interaction between the mite species and the fungus species ($F_{6, 97.5}$=163.91, *P*<0.001). Despite *T. urticae* being the most susceptible species to infection by all isolates, isolate ARSEF2009 caused a similar mortality in *T. urticae* and *N. californicus* (Fig. 2).

1.3.2 Transmission of entomopathogenic fungi by predatory mites

1.3.2.1 Neoseiulus californicus and Tetranychus urticae

When data on adult *T. urticae* mortality were analysed, there were significant differences between the control treatment and the predatory mite treatments (combining clean predators and those vectoring conidia) ($F_{1, 162}$=1638.96, *P*<0.001). Overall mortalities (as proportions dead) were 0.03 (Confidence Intervals (CI) = 0.0290-0.0478) and 0.58 (CI=0.5687-0.6051) for the control and predatory mite treatments, respectively. A significantly greater mortality of *T. urticae* was found in treatments with predatory mites vectoring conidia (0.64, CI=0.6242-0.6736) than in treatments with only predatory mites (0.55, CI=0.5302-0.5790) ($F_{1, 162}$= 36.52, *P*<0.001). When the effect of

N. californicus vectoring conidia was compared amongst the different fungal isolates, significant differences were found ($F_{3, 162}$=33.94, P<0.001). The greatest mortality was found in treatments where *N. californicus* was vectoring *B. bassiana* (Bb88) and *M. anisopliae* (Ma129) conidia compared with the other two isolates (Fig. 3A).

A similar trend was found when the mortality of *T. urticae* nymphs were analysed. Mortality (as proportion dead) in the control treatment was practically non-existent (8.94×10^{-7}, CI=2.02×10^{-14}- 0.9752) compared with the predatory mite treatments (combining clean predators with those vectoring conidia) (0.66, CI=0.6497-0.6730) ($F_{1, 162}$=6808.39, P<0.001). In treatments where predatory mites were vectoring conidia, the mortality (0.69, CI=0.6822-0.7170), was significantly greater than in treatments where only predatory mites were present (0.65, CI=0.6343-0.6675) ($F_{1, 162}$=21.72, P=<0.001). When the effect of *N. californicus* vectoring conidia was compared amongst the different fungal isolates, significant differences were found ($F_{3, 162}$=32.83, P<0.001). The greatest mortality was found when *N. californicus* was vectoring conidia of *M. anisopliae* (Ma129) and *B. bassiana* isolates (Bb88) compared with the other two isolates (Fig. 3B).

The number of viable eggs in each arena was greater ($F_{1, 159}$=7916.82, P<0.001), in the control treatment (644±6.11) compared with the predatory mite treatments (94.5±6.11). The mean number of viable eggs were smaller in the treatment with predatory mites vectoring conidia (81±7.06) compared with treatments where only predatory mites were present (108±7.06) ($F_{1, 159}$=31.59, P<0.001). The fewest eggs were found in treatments where *N. californicus* was vectoring conidia of *M. anisopliae* (Ma129) and *B. bassiana* (Bb88), followed by *I. fumosorosea* (Pfr4) and *L. lecanii* (ARSEF2009), the latter being the treatment with the greatest mean number of viable eggs ($F_{3, 159}$=26.64, P<0.001) (Fig. 3C).

1.3.2.2 Phytoseiulus persimilis and Tetranychus urticae

When data for adult *T. urticae* mortality were analysed, there were significant differences between the control treatment and predatory mite treatments (combining clean predators and those vectoring conidia) ($F_{1, 162}$=1905.17, P<0.001). The overall mortalities (as proportion dead) were 0.03 (CI=0.02522-0.04395) and 0.68 (CI=0.6682-0.70) for the control and predatory mite treatments, respectively. Mortalities in treatments with *P. persimilis* vectoring conidia (0.71, CI=0.6876-0.7371) were greater than in treatments with only *P. persimilis* (0.67, CI=0.6479-0.6963) ($F_{1, 162}$= 6.63, P=0.011). When the effect of the different isolates was analysed, significant differences were found ($F_{3, 162}$=27.70, P<0.001). The greatest mortality was found in treatments where *P. persimilis* was vectoring *B. bassiana* (Bb88) and *M. anisopliae* (Ma129) conidia, compared with the other two isolates (Fig. 4A).

When data obtained from *T. urticae* nymphs were analysed, the mortality (as proportion dead) in the control treatment was very low (3.28×10^{-7}, CI=4.35×10^{-19}- 0.9999) compared with mortality in treatments with predatory mites (combining clean and vectoring conidia) (0.75, CI=0.7391-0.7620) ($F_{1, 162}$=9084.54, P<0.001). A greater proportion of mortality was obtained in treatment using *P. persimilis* vectoring conidia (0.78, CI=0.7701-0.8000) compared with treatments where only *P. persimilis* was used (0.73, CI=0.7247-0.7536) ($F_{1, 162}$=23.10, P<0.001). When a comparison amongst predatory mites vectoring the different isolates was done, significant differences were found ($F_{3, 162}$=34.08, P<0.001). The greatest mortality was found in treatments where *P. persimilis* was vectoring *B. bassiana* (Bb88) and *M. anisopliae* (Ma129) conidia, compared with the other two isolates (Fig. 4B).

The number of viable eggs in each arena was greater ($F_{1, 159}$=6318.63, P<0.001), in the control treatment (642±6.94) compared with the predatory mite treatments (69.5±6.94). There were fewer viable eggs in the treatment with predatory mites vectoring conidia (59.1±8.02) compared with treatments where only predatory mites were present (80.0±8.02) ($F_{1, 159}$=12.13, P=0.010). The fewest eggs were found in treatments where *P. persimilis* was vectoring conidia of *M. anisopliae* (Ma129) and *B. bassiana* (Bb88), followed by *I. fumosorosea* (Pfr4) isolate and *L. lecanii* (ARSEF2009); the latter was the treatment with the greatest mean number of viable eggs ($F_{3, 159}$=9.83, P<0.001) (Fig 4C).

1.4 Discussion

In order to evaluate the combined use of predatory mites and entomopathogenic fungi, we first evaluated the susceptibility of *T. urticae* and two predatory mites, *N. californicus* and *P. persimilis*, to fungal pathogens. Overall, all mite species evaluated were susceptible to infection. However, *T. urticae* was the most susceptible species (Fig. 2). Wu et al. 2017 proposed that the greater hardness and stiffness of the cuticle of *Neoseiulus barkeri* Hughes compared with the cuticle of *T. urticae* was responsible for the lower susceptibility of *N. barkeri* to infection by *B.* Although we did not compare the hardness of the cuticle of the mite species we studied, we believe this might contribute to our results. Interestingly, our results also showed that outcomes varied depending on the fungal species. Susceptibility to *L. lecanii* isolate ARSEF2009 was similar in *N. californicus* and *T. urticae*. Wu et al. 2017, also reported that susceptibility of *N. barkeri* to infection may increase if the conidia became attached to the ventral surface, where the cuticle was softer (and similar to *T. urticae*) compared with the dorsal surface. We believe, the sticky nature

of the *L. lecanii* conidia may have allowed conidia to attach to the ventral surface causing greater infection in *N. californicus* compared with the other isolates (Fig. 2). It is unclear why this was not observed in *P. persimilis*. Nevertheless, based on our results, we believe that all isolates, with the potential exception of *L. lecanii*, could be used in combination with predatory mites to reduce *T. urticae* populations. For this reason we used all isolates in the transmission experiments.

Effective targeting of *T. urticae* using conidial suspensions is often ineffective because the plant structure prevents conidia from reaching the pest. In the long term, this may increase costs related to the control of this pest using entomopathogenic fungi because the number of applications necessary would increase. Combined use of predatory mites and entomopathogenic fungi remains less studied, but has great potential for more efficient biological control (Lin et al. 2017) and is what we evaluated in this study.

Overall, our results showed greater mortality in treatments where nymphs and adults of *T. urticae* were exposed to predatory mites vectoring conidia compared with treatments with predatory mites alone; the greatest mortality was consistently achieved in *B. bassiana* and *M. anisopliae* treatments (Figs. 3 and 4). These isolates also caused the greatest mortality in *T. urticae* when applied directly under laboratory conditions (Fig. 2). In contrast, the *L. lecanii* isolate (ATTC2009), which caused a similar mortality as *B. bassiana* and *M. anisopliae* when applied directly (Fig. 2), did not achieve as high a mortality as *B. bassiana, M. anisopliae* or *I. fumosorosea* when vectored by predatory mites. Mortality is evidently related to the number of conidia acquired by the predatory mites (Table 1) and our results showed that predatory mites acquired more *B. bassiana* and *M. anisopliae* conidia than *I. fumosorosea* and *L. lecanii*; the smallest number of conidia acquired were of *L. lecanii* (Table 1). It is unclear why fewer *L. lecanii* conidia were acquired by the predatory mites compared with *B. bassiana* or *M. anisopliae* isolates, as all isolates

were treated similarly. We expected a similar number of *L. lecanii* conidia, or even more, to be attached to the predatory mites, due to the sticky nature of the *L. lecanii* conidia which may have meant more *L. lecanii* conidia would adhere to mite cuticles. However, it is also possible that the sticky nature of the *L. lecanii* conidia meant they formed conglomerates on mite legs prompting grooming behaviour that would have reduced the number of conidia. Grooming behaviour has been reported previously for *P. persimilis* (Ullah and Lim 2017; Wu et al. 2018), although these authors reported grooming behaviour in response to exposure to *B. bassiana*, which suggests that grooming may have occurred in all our fungal treatments. It is possible that the effect was strongest for *L. lecanii*. The dry conidia of *B. bassiana* and *M. anisopliae* are also small, which may mean that they are more easily transported by a vector, as demonstrated by Al-Mazra'Awi et al. (2007) and Butt et al (1998). Vectoring conidia had no significant negative effect of the predatory mites. During the 9 days of the experiment, only 3 - 13% of the predatory mites died (Table 1); only in the *B. bassiana* and *M. anisopliae* treatments did the dead predatory mites sporulate (data not shown). Also, we observed nymphs and eggs of the predatory mites in the arenas suggesting that they were reproducing and developing, confirming limited negative effects of vectored conidia.

We did not observe differences in *T. urticae* mortality between treatments with *N. californicus*, a generalist predator (Fig. 3), and *P. persimilis*, which is considered as a specialist predator (Fig. 4). The lower susceptibility of both predatory mites to fungal infection compared with *T. urticae* (Table 1) suggests that both species could be used successfully in combination with entomopathogenic fungi to achieve greater mortality in *T. urticae* than when they are used alone. We cannot separate the effect of infection from predation in the dual treatments because most of the cadavers had both signs of predation and the presence of mycelium. This could represent either mortality by predation with the fungus growing as a saprophyte, or mites that had first become

infected and then been fed on by predatory mites. It would be necessary to test this in a different experiment, in order to determine whether intraguild predation was occurring. Furthermore, we evaluated both predators under controlled conditions; our results need to be confirmed under greenhouse and field conditions when both natural enemies would be subject to varying environmental conditions and interacting with other arthropods, including other mite species.

In conclusion, *T. urticae* was more susceptible to fungal infection than *N. californicus* and *P. persimilis*. The *B. bassiana* and *M. anisopliae* isolates used in this study caused the greatest infection rates compared with the isolates of *I. fumosorosea* and *L. lecanii* we used. No differences were found in *T. urticae* mortality caused by either of the two predatory mite species; greatest mortality was achieved when predatory mites were vectoring *B. bassiana* or *M. anisopliae* conidia. A combination of either predatory mite with either *B. bassiana* or *M. anisopliae* represents a strategy with great potential for the biological control of *T. urticae*. However, before practical recommendations can be made, we need to confirm our results under greenhouse and field conditions.

1.5 Author Contribution Statement

OCR, AWGF, MTSG and FTM conceived and designed the research. OCR conducted the experiments. OCR and AWFG analysed the data. MTSG and FTM contributed with reagents and equipment. OCR and AWGF wrote the manuscript. All authors have read and approved the manuscript.

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1.8 Compliance with ethical standards

Conflict of interest: The authors declare that they have no financial/commercial conflicts of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Tables

Table 1: Mean number of conidia (± SD) per mite acquired after exposure to a fungal source for 12 h. Total number of predatory mites used in the complete experiment/number of mites that died at the end of experiment. Dead predatory mites sporulated only in treatments with *B. bassiana* and *M. anisopliae*.

Isolate	*N. californicus*		*P. persimilis*	
	Conidia/mite	Total - infected	Conidia/mite	Total - infected
B. bassiana (Bb88)	51.70 ± 7.16	60/8	70.75 ± 17.95	60/4
M. anisopliae (Ma129)	72.96 ± 10.14	60/3	76.25 ± 12.32	60/4
I. fumosorosea (Pfr4)	33.38 ± 7.32	60/2	66.23 ± 13.98	60/2
L. lecani (ATTC2009)	22.57 ± 7.74	60/4	25.61 ± 6.09	60/2

Figures

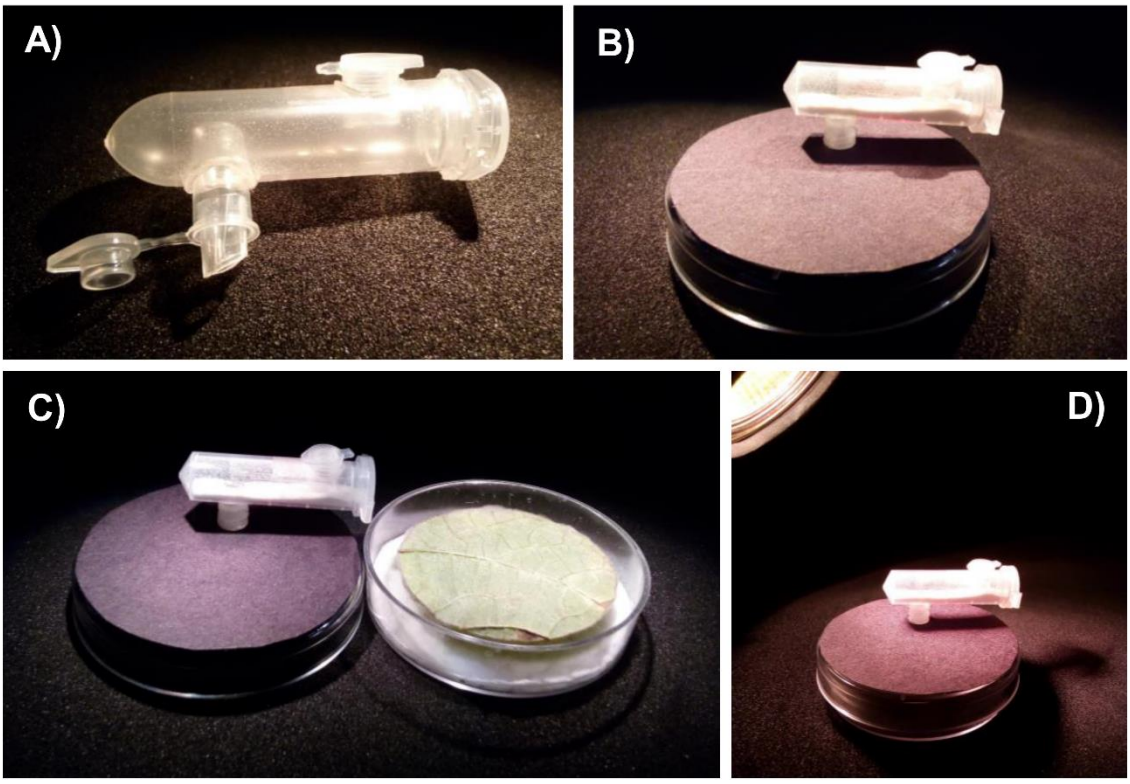

Figure 1: Device developed for the contamination of predatory mites and introduction into arenas containing *T. urticae* adults. A) Modified 2 mL Eppendorf tube, B) Eppendorf tube with 500 mg of conidia and mycelium, C) Eppendorf tube attached to the experimental arena, and D) Device exposed to a heat source to force predatory mites to move into the arena.

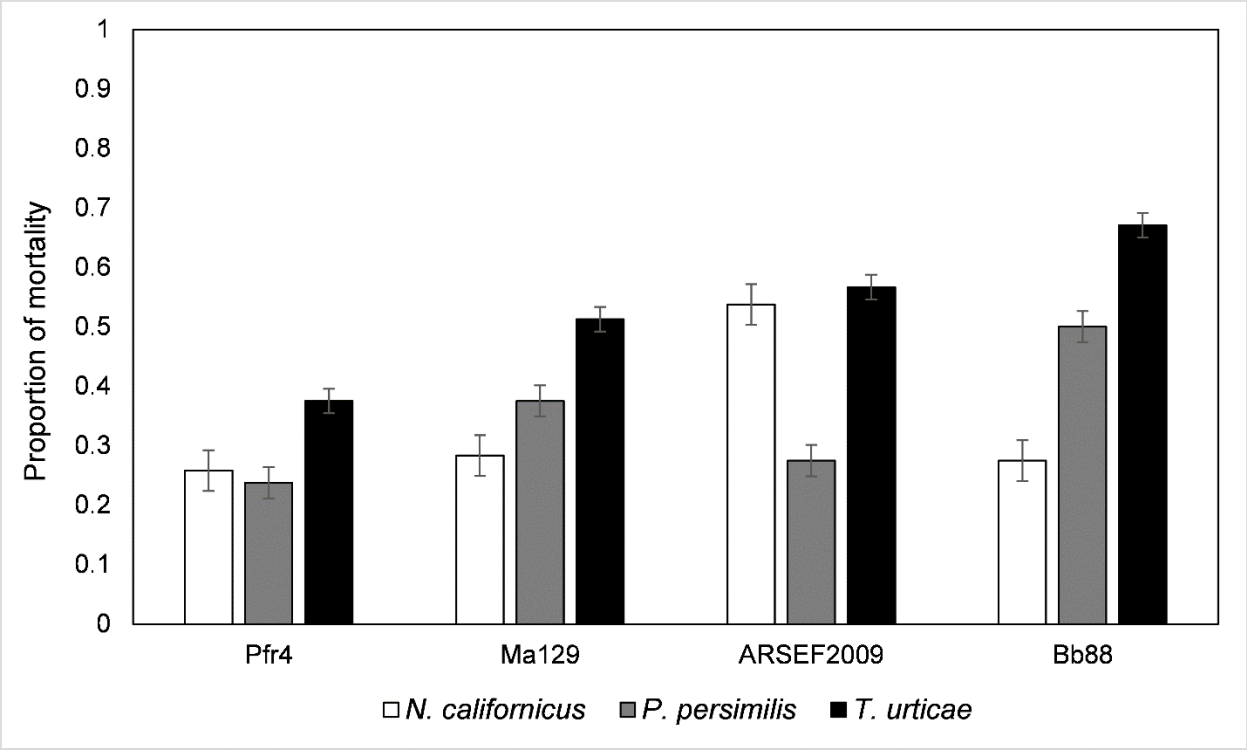

Figure 2: Proportions of *Tetranycus urticae*, *Phytoseiulus persimilis* and *Neoseiulus californicus* adults infected by four fungal isolates. Error bars represent ± 1×SEM.

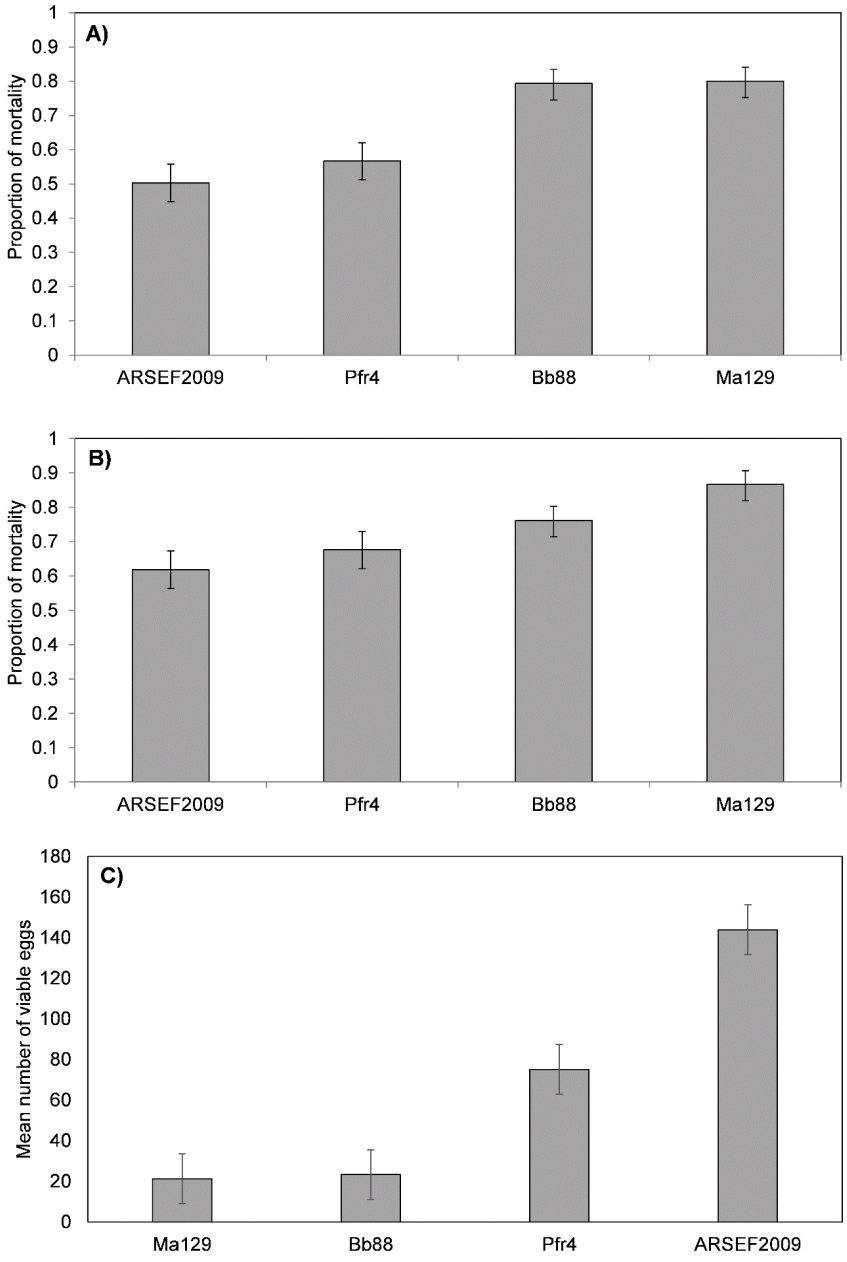

Figure 3: Proportion of mortality achieved in adults (A) and nymphs (B) of *T. urticae*. Number of eggs of *T. urticae* (C) when exposed to *N. californicus* vectoring the conidia of four fungal isolates. Error bars in A and B represent 95% confidence intervals back-t transformed from the logistic scale. Error bar in C represents ±1 × SEM (df = 170).

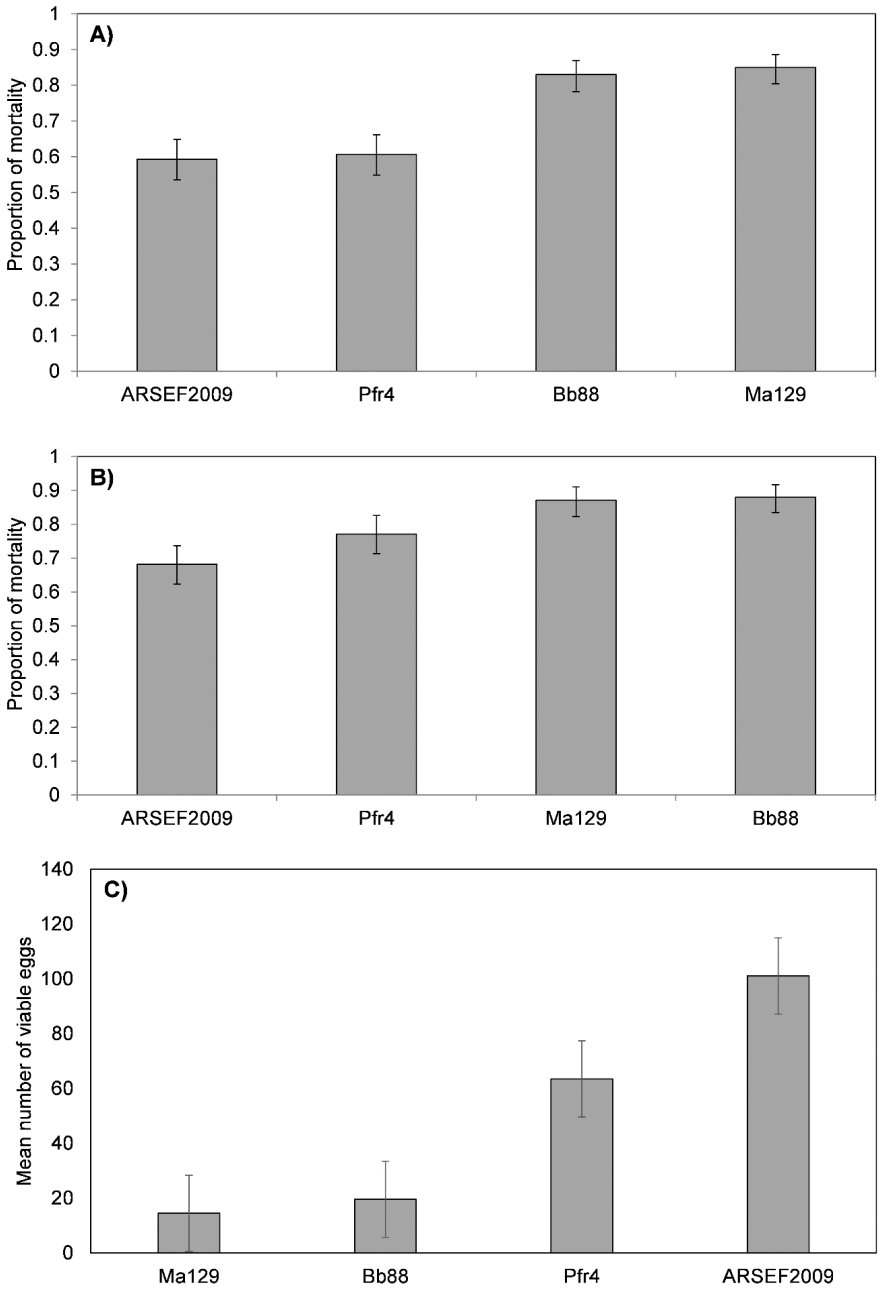

Figure 4: Proportion of mortality obtained in adults (A) and nymphs (B) of *T. urticae*. Number of eggs of *T. urticae* (C) when exposed to *P. persimilis* vectoring conidia of four fungal isolates. Error bars in A and B represent 95% confidence intervals back-transformed from the logistic scale. Error bar in C represents ±1 × SEM (df = 170).

DISCUSIÓN GENERAL

Tetranychus urticae es una de las especies fitófagas más importantes en cultivos como la frambuesa, fresa y zarzamora, por ello, se evaluó la patogenicidad de diferentes aislamientos de hongos entomopatógenos sobre adultos de este ácaro. Asimismo, se comparó la susceptibilidad de adultos de sus principales depredadores asociados: *Phytoseiulus persimilis* y *Neoseiulus californicus*, a dichos entomopatógenos. Las especies contempladas fueron: *Beauveria bassiana, Metarizhium anisopliae, Isaria fumosorosea y Lecanicillium lecanii*; y las tres especies de ácaros descritas anteriormente. Se observó una mayor susceptibilidad de *T. urticae* a la infección por hongos entomopatógenos en comparación con *N. californicus* y *P. persimilis*. *Beauveria bassiana* fue la especie que causo mayor proporción de mortalidad en *T. urticae*, seguido de *L. lecanii* y *M. anisopliae.* En el caso de *N. californicus*, *L. lecanii* fue la especie que le causó mayor proporción en su mortalidad. En *P. persimilis* fue *B. bassiana*. La variación en la mortalidad obtenida se podría atribuir a las propiedades cuticulares específicas de cada especie de ácaro evaluada. De acuerdo con Wu *et al.* (2018), el género *Neoseiulus* presenta placas esclerosadas en la zona dorsal del cuerpo, las cuales se sugiere podrían reducir considerablemente el riesgo de ser infectado por hongos entomopatógenos, en comparación con *T. urticae.* El hecho de que los ácaros depredadores hayan sido menos susceptibles a la infección sugiere que podrían ser candidatos idóneos para ser aplicados en conjunto con los hongos entomopatógenos. Para ello, fue elaborado un dispositivo de impregnación de conidios en formulación sólida (polvo), para facilitar la adhesión al cuerpo del artrópodo transmisor (Al Mazra'Awi *et al.* 2007). Los resultados obtenidos muestran que los tratamientos donde se usaron ácaros depredadores contaminados con conidios ocasionaron la

mayor mortalidad, en comparación con los tratamientos donde únicamente se usaron los ácaros depredadores sin conidios.

Por otro lado, no se observó una diferencia entre la capacidad de *P. persimilis* y *N. californicus* para actuar como vectores de esporas de hongos entomopatógenos. Sin embargo, si observamos que la mayoría de los adultos de *T. urticae* muertos presentaron crecimiento micelial externo proveniente de las especies de entomopatógenos, lo cual sugiere que el hongo se podría haber desarrollado de manera saprofita sobre un ácaro muerto o que los ácaros depredadores se alimentaron de adultos de *T. urticae* previamente infectados, lo que sugiere una depredación intragremial.

Al momento del diseño del experimento, se esperaba que al ácaro depredador especialista ocasionara una mayor mortalidad de *T. urticae* comparado con el generalista, debido a que este tendría una mejor estrategia de búsqueda de la presa. Sin embargo, los resultados no mostraron diferencias. Con base en estos resultados, ambas especies de ácaros depredadores podrían usarse en combinación con *B. bassiana* o *M. anisopliae* para el control efectivo de *T. urticae*. Sin embargo, es necesario validar estos resultados bajo condiciones de campo, lo que implicaría la presencia de otros artrópodos y condiciones adversas, lo que posiblemente haría evidente las diferentes habilidades de ambos depredadores para cazar a su presa.

En conclusión, *T. urticae* es la especie más importante en frutillas, es más susceptible a la infección por hongos entomopatógenos, especialmente *B. bassiana* y *M. anisopliae*, en comparación con los ácaros depredadores. Los tratamientos de ácaros depredadores en combinación con conidios de hongos ocasionaron mayores mortalidades de *T. urticae* en comparación con únicamente depredadores; sin diferencias entre especies de ácaros depredadores. Es necesario realizar estudios en invernadero y campo para confirmar los resultados.

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