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INTERACCIONES ENTRE HONGOS ENTOMOPATÓGENOS Y *Tamarixia radiata* (HYMENOPTERA: EULOPHIDAE) EN POBLACIONES DE *Diaphorina citri* (HEMIPTERA: LIVIIDAE)

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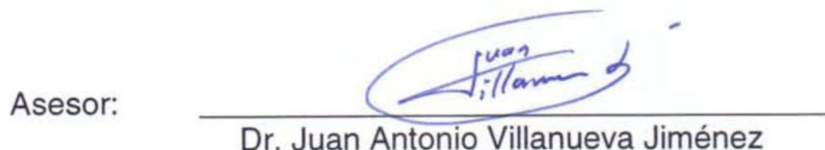
Asesor: 
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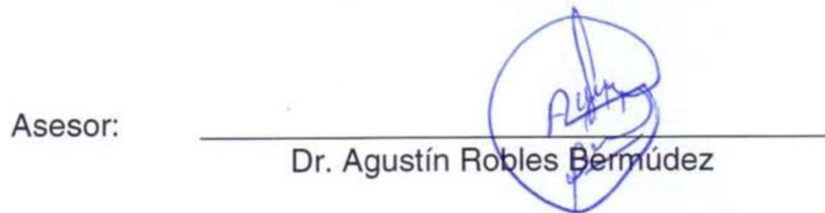
Asesor: 
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INTERACCIONES ENTRE HONGOS ENTOMOPATÓGENOS Y *Tamarixia radiata* (HYMENOPTERA: EULOPHIDAE) EN POBLACIONES DE *Diaphorina citri* (HEMIPTERA: LIVIIDAE)

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Resumen

El psílido asiático de los cítricos *Diaphorina citri* es una plaga de importancia económica mundial debido a que es el vector de la enfermedad del Huanglongbing. En México, como estrategia de control biológico, se utilizan el parasitoide *Tamarixia radiata* y los hongos entomopatógenos *Isaria javanica* y *Metarhizium anisopliae*. El hongo *Hirsutella citriformis* se encuentra regulando de forma natural las poblaciones de *D. citri* en las huertas citrícolas de nuestro país. No obstante, cuando se introducen más de dos organismos para el control de una plaga, es necesario conocer que ocurre cuando los agentes de control biológico compiten por un mismo huésped. Para este trabajo se realizaron experimentos *in vitro* donde se estudió la susceptibilidad de *D. citri* y el parasitoide *T. radiata* a seis aislamientos, dos de cada una de las especies *Beauveria bassiana*, *Metarhizium anisopliae* e *Isaria fumosorosea,* y qué ocurre cuando ambos enemigos naturales interaccionan. Primeramente, se realizaron experimentos para evaluar la susceptibilidad de ninfas y adultos de *D. citri,* y adultos de *T. radiata* a los hongos entomopatógenos y se calculó la CL_{50}. Se encontró que las ninfas son más susceptibles a la infección de los hongos que los adultos de *D. citri*; mientras que en *T. radiata,* la susceptibilidad fue menor que las ninfas de *D. citri*. Posteriormente, con los aislamientos más virulentos, B1 de *B. bassiana* y MA129 de *M. anisopliae*, se estudió el efecto del órden de llegada en la interacción de estos aislamientos en el parasitoide en ninfas de *D. citri*. Cuando las ninfas de *D. citri* primero se inocularon con los hongos y posteriormente se expusieron con diferentes tiempos de infección fúngica al parasitoide *T. radiata*, se observó que la parasitación disminuye a las 72 h y la depredación de las ninfas por la hembra adulta de *T. radiata* fue menor en 24 h posteriores a la inoculación del hongo en comparación con 0 y 72 h. Por otra parte, cuando las ninfas contenían diferentes estadios de desarrollo del parasitoide (huevo, larva y pupa) y se inocularon con los aislamientos B1 y Ma129 se encontró que el estado de pupa es el menos susceptible a la infección de hongos, además de tener la mayor emergencia del adulto. FInalmente, se investigó la susceptibilidad de ninfas y adultos de *D. citri* a la infección del hongo *H. citriformis*, así como la transmisión del hongo de adultos a ninfas. Las ninfas de *D. citri* fueron más susceptibles a la infección de *H. citriformis* que los adultos. No se encontró evidencia de transmisión de *H. citriformis* de adultos a ninfas. De acuerdo a los resultados obtenidos en este trabajo, se sugiere la posibilidad de combinar los aislamientos B1 y MA129 y el parasitoide *T. radiata,* para el control biológico de *D. citri* en poblaciones de campo.

Palabras clave: *Diaphorina citri*, *Tamarixia radiata*, susceptibilidad, interacción, hongos entomopatógenos.

INTERACTIONS BETWEEN ENTOMOPATHOGENIC FUNGI AND *Tamarixia radiata* (HYMENOPTERA: EULOPHIDAE) IN POPULATIONS OF *Diaphorina citri* (HEMIPTERA: LIVIIDAE)

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Abstract

Diaphorina citri is the vector of the disease known as the citrus Huanglongbing. In Mexico, as part of a biological control program, the ectoparasitoid *Tamarixia radiata* and the entomopathogenic fungi *Isaria javanica* and *Metarhizium anisopliae* are currently used. In addition, the fungus *Hirsutella citriformis* has been found infecting field populations of *D. citri* in citrus orchards in Mexico. When more than one natural enemy is used, it is important to know the outcomes of their potential interactions when competing for the same host, in order to use them more efficiently. We performed a series of *in vitro* experiments to study the susceptibility of *D. citri* and *T. radiata* against six isolates, two of each of the species *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea,* and the interactions of these natural enemies in *D. citri* populations. Firstly, dose-response experiments were done using the six isolates against both nymphs and adults of *D. citri* and *T. radiata*. Results showed that nymphs were more susceptible against fungal infection than adults of *D. citri* and the parasitoid. Secondly, using the most virulent isolates of *B. bassiana* (B1) and *M. anisopliae* (Ma129), we studied the effect of prior residency in their interactions. When *D. citri* nymphs with different fungal infection times were exposed to the parasitoids, parasitism was highly reduced in nymphs with 72 h post inoculation in comparison with nymphs with 0 and 24 h of fungal infection; host feeding by the parasitoids was reduced in nymphs with 24 h of fungal inoculation. No effect of isolate was observed. When nymphs carrying parasitoids with different developmental stages (egg, larvae and pupae) were fungal inoculated, nymphs carrying pupae of the parasitoid showed the greater adult parasitoid emergence. Finally, when the susceptibility of nymphs and adults of *D. citri* against infection by *Hirsutella citriformis* was investigated, nymphs were more susceptible than adults; however, when nymphs were exposed to fungal contaminated *D. citri* adults, only adults were infected suggesting no transmission between adults and nymphs could be achieved. We propose the combined use of entomopathogenic fungi, specifically isolates B1 and MA129, and *T. radiata* for the biological control of field populations of *D. citri*.

Key words: *Diaphorina citri*, *Tamarixia radiata*, susceptibilidad, interaction, entomopathogenic fungi.

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Capítulo 1. Introducción General

México es un productor líder de cítricos ya que se ubica en el quinto lugar a nivel mundial. Los cítricos que tienen importancia económica para la agricultura mexicana son: limón, mandarina, naranja y toronja. Estos frutales se producen en 23 estados de la República y los estados con mayor producción son Veracruz, San Luis Potosí, Michoacán, Tamaulipas, Colima y Nuevo León, donde Veracruz es el que tiene la mayor producción con 2,987,973 toneladas (**Salcedo et al., 2010; SAGARPA, 2012; SIAP, 2014,**). Sin embargo, la citricultura mexicana se encuentra amenazada por la enfermedad del Huanglongbing (HLB) o dragón amarillo de los cítricos, debido a que todas las especies comerciales de cítricos son susceptibles a dicha enfermedad (**Bové, 2006; Halbert y Manjunath, 2004; Trujillo-Arriaga, 2010**). La enfermedad del HLB está asociada con tres bacterias estrictas del floema *Candidatus* Liberibacter asiaticus en Asia y América, *Ca*. L. africanus en Africa y *Ca*. L. americanus en Brazil y Sudámerica (**Gottwall, 2010; Jagoueix et al., 1994**). Se transmite por dos vectores, el psílido asiático de los cítricos *Diaphorina citri* (Kuwayama) en Asia y América y *Trioza erytreae* (Del Guercio) en África (**Bové, 2006; Jagoueix et al., 1994; Teixeira, 2005**).

Los daños que causan las ninfas y adultos de *D. citri* son directos e indirectos. El daño directo ocurre cuando las poblaciones son altas (**Aubert, 1987**). El insecto, extrae la savia de las plantas lo que las debilita, así mismo, promueve la formación de fumagina en el follaje que crece en las excreciones del insecto. Además, durante su alimentación inyectan toxinas a la planta que detienen el crecimiento de los brotes y deforman las hojas (**Grafton-Cardwell et al., 2006; Michaud, 2004**).

El daño indirecto es el más importante por la capacidad de transmitir la bacteria que causa la enfermedad del HLB (**Michaud, 2004**), que provoca que los árboles de cítricos infectados con HLB sean improductivos a partir de 5 a 10 años (**Aubert et al., 1996; Roistacher 1996**). Los síntomas de la enfermedad se observan en las hojas como un moteado difuso, hojas de color amarillas, engrosamiento y aclaramiento de nervaduras y hojas con apariencia corchosa. En los frutos con HLB hay reducción del tamaño, son asimétricos, de mayor espesor y con reverdecimiento de la cáscara, aumenta la acidez, se presenta una inversión de color de maduración, aborto de semillas y caída prematura de los mismos (**Bové, 2006**).

Actualmente, los programas de manejo integrado del vector *D. citri* y la enfermedad del HLB incluyen acciones de control químico, biológico y cultural (**Hall et al., 2011**). Diversos estudios reportan que el control biológico es una estrategia potencial para el control de *D. citri*; sin embargo, el control químico es el más utilizado (**Hall, 2008**). En México, para el control del HLB y su vector *D. citri* la Dirección General de Sanidad Vegetal, está implementando el programa de Áreas Regionales de Control (ARCO), donde la superficie mínima para un ARCO es de1,000 ha donde se realizan actividades como monitoreo del vector con trampas amarillas, control químico y control biológico (**Robles-García, 2012**). Las liberaciones del parasitoide *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) que tiene cierta especificidad sobre *D. citri*, se llevan a cabo a partir del año 2010 a la fecha en varios estados citrícolas en zonas urbanas y huertos abandonados de cítricos con el fin de incrementar las poblaciones naturales del parasitoide (**Arredondo-Bernal et al., 2010**).

Adicionalmente, se hacen aplicaciones de los hongos entomopatógenos *Isaria javanica* (Frieder. & Bally) Samson & Hywell-Jones y *Metarhizium anisopliae* s.l. (Metsch.) Sorokin para disminuir poblaciones del insecto vector en huertos comerciales (**Arredondo-Bernal et al., 2010; Ayala Zermeño et al., 2015**).

El que *D. citri* se desarrolle en cultivos perennes permite que exista una diversidad de enemigos naturales de las familias Chrysopidae, Coccinellidae y Mantidae, y del orden Araneae que se encuentran regulando las poblaciones de *D. citri* de forma natural (**Chan Teck et al., 2011; Lozano Contreras y Jasso Argumedo, 2012**).

Los hongos entomopatógenos son enemigos naturales de especies de insectos y ácaros que aportan beneficios a los ecosistemas, incluyendo el control de plagas agrícolas (**Hoy et al., 2010**). Existen trabajos donde se menciona que los hongos *Beavueria bassiana* (Balsamo) Vuillemin, *Hirsutella citriformis* Speare, *Isaria. fumosorosea* Wize y *M. anisopliae* son patógenos de *D. citri* (**Avery et al., 2011; Ferreira Pinto et al., 2012; Orduño-Cruz et al., 2015**).

El parasitoide *T. radiata* tiene la capacidad de depredar y parasitar su húesped, lo cual le permite que una hembra adulta matar hasta 500 ninfas de *D. citri* durante toda su vida. Cuando se trata de parasitar, prefiere ninfas de quinto instar y para depredar selecciona ninfas de los primeros instares, aunque tiene inclinación por ninfas de tercer instar (**Hoy et al., 2006**).

En la actualidad, en los huertos citrícolas de nuestros país, *D. citri* está coexistiendo principalmente con el parasitoide *T. radiata* y los hongos entomopatógenos *I. javanica, H. citriformis y M. anisopliae* (**Arredondo-Bernal *et al.*, 2010; Ayala-Zermeño et al., 2015; KHIC observación personal**).

Lo anterior sugiere que la posible actividad combinada de estos enemigos naturales podría reducir las poblaciones de *D. citri.* Sin embargo, cuando se establecen una o más de estas estrategias adicionales a las que actualmente se usa para el manejo de este insecto, debe estar soportado por estudios que aporten información básica acerca del tipo de interacciones que podría suceder entre estos enemigos naturales en el control de *D. citri*. El conocimiento de las interacciones, relacionadas con la regulación de una plaga por enemigos naturales o agentes de control biológico, permite diseñar las mejores estrategias dentro de un programa de manejo integrado de plagas.

Por lo anterior dentro de esta investigación se propuso como objetivo general:

El estudiar las interacciones de los hongos entomopatógenos *B. bassiana, I. fumosorosea, M. anisopliae* e *H. citriformis* con el parasitoide *T. radiata* en poblaciones de *D. citri*.

Para ello se plantearon los objetivos particulares siguientes:

- Estimar la virulencia de los hongos entomopatógenos en *Diaphorina citri* (Hemiptera: Liviidae) y su parasitoide *Tamarixia radiata* (Hymenoptera: Eulophidae) en condiciones de laboratorio.
- Evaluar las interacciones entre los hongos entomopatógenos y *Tamarixia radiata* (Hymenoptera: Eulophidae) en poblaciones de *D. citri* (Hemiptera: Liviidae).
- Estimar la susceptibilidad de ninfas y adultos de *D. citri* a el hongo entomopatógeno *H. citriformis*.

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Capítulo 2. Virulence of entomopathogenic fungi against *Diaphorina citri* (Hemiptera: Liviidae) and its parasitoid *Tamarixia radiata* (Hymenoptera: Eulophidae) under laboratory conditions

Abstract

The Asian citrus psyllid, *Diaphorina citri*, is a global pest of citrus that transmits the bacteria associated with the citrus greening disease, Huanglongbing. Entomopathogenic fungi and the parasitoid *Tamarixia radiata* are amongst the most important biological control agents of this pest. It is very likely that these organisms will interact with each other in *D. citri* populations. In evaluating these potential interactions, the virulence of six fungal isolates (*Beauveria bassiana s.l.* isolates B1 and B3; *Metarhizium anisopliae s.s.* isolates Ma129 and Ma65, and *Isaria fumosorosea* isolates I2 and Pae) were compared against nymphs and adults of *D. citri*, and adults of the parasitoid *T. radiata*. Dose response assays using a series of conidial concentrations (1 x 10^4 to 1 x 10^8 conidia mL^{-1}) were conducted against all three groups. Results showed that *D. citri* nymphs were more susceptible to fungal isolates than *D. citri* adults; probit analysis showed that isolate Ma129 was the most virulent against nymphs and B1 the most virulent against adults. Isolate B1 was also the most virulent against the parasitoid, *T. radiata*. The virulence of isolates Ma129 and B1 when estimated against *T. radiata* were lower when compared to nymphs and adults of *D. citri*. The impact of our results on the potential interaction between these two biological control agents in *D. citri* populations is discussed.

Keywords: *Beauveria bassiana*, *Metarhizium anisopliae*, *Isaria fumosorosea,* probit analysis.

2.1 Introduction

The citrus industry has been severely affected by Huanglongbing (HLB); a disease associated with the bacteria *Candidatus* Liberibacter spp. This pathogen is transmitted by the psyllid *Diaphorina citri* (Kuwayama) (**Bové, 2006**). In Mexico, HLB was first detected in 2009 in the State of Yucatan and currently *D. citri* populations have been reported in all citrus production regions in Mexico (**Trujillo-Arriaga, 2010**). Although the use of chemical pesticides is the most common control strategy, biological control has also been attempted in Mexico using the eulophid parasitoid *Tamarixia radiata* (Waterston), the entomopathogenic fungi *Isaria fumosorosea* Wize and *Metarhizium anisopliae sensu lato* (Metsch.) Sorokin (**Trujillo-Arriaga, 2010**); however, there are no formal reports on the actual impact of these biological control strategies on field populations of *D. citri*. Entomopathogenic fungi have attributes that make them strong candidates for biological control of this pest. For example, their infection capacity against *D. citri* (**Orduño-Cruz et al., 2015a**) and the relative ease of mass production, formulation and field release (**Moore, 1993; Butt Jackson and Magan, 2001; Shah and Pell, 2003**). **Orduño-Cruz et al., (2015a, b)** described *in vivo* and *in vitro* and experiments to select fungal isolates for biological control of *D. citri*.

Thus, the infection capacity of *Beauveria bassiana sensu lato*, (Bals.-Criv.) Vuill, *M. anisopliae sensu stricto, I. fumosorosea* and *Hirsutella citriformis* Speare against adult *D. citri* has been demonstrated under laboratory conditions (**Avery et al., 2009; Avery et al., 2011; Avery et al., 2012; Hall et al., 2012; Gandarilla-Pacheco et al., 2013; Orduño-Cruz et al., 2015a**). However, once these isolates are released in the field,

they are likely to interact with the parasitoid *T. radiata*, and the outcomes of these interactions have not been studied.

During the development of fungal isolates for use in a biological control programme, any effects on non-target organisms, such as parasitoids and predators, are amongst the most important things to consider (**Posada and Vega, 2005**). We believe that estimating the relative virulence of the previously-studied fungal isolates (**Orduño-Cruz et al., 2015a, b**) against both *D. citri* and *T. radiata* is an important first step before studying other direct and indirect interactions between these two organisms.

This would allow us to understand the potential outcomes of the interaction between these two species in *D. citri* populations. To achieve this, a series of experiments was done to estimate the virulence (LC_{50}) of six fungal isolates, two from each of the species *B. bassiana s.l.*, *M. anisopliae s.s.* and *I. fumosorosea*, against adults of *T. radiata*, and adults and nymphs of *D. citri*.

2.2 Material and Methods

2.2.1 Fungal isolates and insect colonies

Six fungal isolates were used (Table 2.1), all of which have been deposited in the Culture Collection of the Insect Pathology Laboratory, Colegio de Postgraduados, Campus Montecillo, Texcoco, Mexico.

For all bioassays nymphs and adults of *D. citri* and adults of *T. radiata* were provided by the Centro Nacional de Referencia en Control Biológico (CNRCB), SENASICA, Tecoman, Colima, Mexico. Nymphs of *D. citri* were used to maintain the *T. radiata* colony. Both colonies were maintained under greenhouse conditions at the CNRCB facilities with 27 ± 2 °C, at 60-80% RH.

Table 2.1 List of isolates used in this study.

Species	Isolate	Host	Reference
Beauveria bassiana s.l.	B3	*Bactericera cockerelli*	Orduño-Cruz et al., 2015b
	B1	*Diaphorina citri*	""
Metarhizium anisopliae s.s.	Ma65	*Aneolamia albofasciata*	Ibarra-Cortés et al., 2013; Orduño-Cruz et al., 2015b*
	Ma129	*Tetranychus urticae*	Ibarra-Cortés et al., 2013
Isaria fumosorosea	Pae	Commercial isolate	Ibarra-Cortés et al., 2013
	I2	*Diaphorina citri*	Orduño-Cruz et al., 2015b

* This isolate was also used by Orduño-Cruz et al., (2015b), but with the isolate code M2.

The *D. citri* colony was maintained on one-year-old *Murraya paniculata* (L.) Jack plants inside steel cages (70×70×70 cm) covered with insect proof mesh. Plants containing mostly 4th instar nymphs were selected; from these, leaflets bearing large numbers of nymphs were removed, placed in an icebox and transported to the laboratory, where groups of 15 nymphs were separated using a fine camel hair brush under the stereomicroscope and placed in the experimental units (60 mm diameter Petri dishes). For *D. citri* adults, insects (~7d old) were captured from the cages using a mouth aspirator and placed, in groups of 15, inside the glass vials (27 mm diameter and 50 mm height) with 1 mm orifices in the lid to allow ventilation. Vials were then placed in an icebox and transported to the laboratory.

Adult parasitoids were collected from *M. paniculata* plants containing parasitized 4th and 5th instar nymphs (parasitoids in the pre-pupal stage). Leaflets were removed and placed in an icebox and transported to the laboratory where they were incubated in darkness at 27 °C at 60% RH until adult parasitoid emergence. Parasitoids were

collected using a mouth aspirator and deposited in glass vials, in groups of 15, as described above and kept in an icebox until needed.

2.2.2 Production of conidial suspensions

Monosporic versions of each isolate were used throughout, and each isolate was not sub- cultured more than three times before retrieving a new vial from -80 °C storage.

Each isolate was cultured on Sabouraud dextrose agar (SDA) in 90 mm diameter Petri dishes. Inoculated SDA dishes were incubated at 25 °C in complete darkness for 20 days until sporulation was apparent. Under sterile laminar flow conditions conidia from each isolate were harvested using a sterile steel spatula to scrape them into 30 mL glass tubes with 20 mL of 0.03 % Tween 80 solution. Conidial suspensions of each isolate were vortexed for 5 min and then filtered using sterile muslin cloths into new 30 mL glass tubes. From each stock suspension, 10 µL were taken, suspended in 990 µL of 0.03 % Tween 80 solution and used to estimate conidial concentration of the stock suspension. Material from the stock suspensions were then serially diluted to produce a range of five different conidial concentrations: 1×10^4, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia mL^{-1}.

All conidial suspensions were maintained at 4 °C for no longer than 24 hours prior to use. Germination in conidial suspensions was assessed prior to experiments, and was always above 95%.

2.2.3 Virulence assays

Experimental design for assays of fungi against *Diaphorina citri* and *Tamarixia radiata*

All insects were inoculated in groups of 15 using a spray tower (glass cylinder of 51 cm diameter and 20 cm height) fitted with a cone spray nozzle (Spraying Systems Co.

Wheaton, IL, USA) attached to an air compressor at 20 psi. Nymphs of *D. citri*, adults of *D. citri* and adults of *T. radiata* were assayed separately, but all isolates were applied against each target on the same day.

For each isolate, groups of 4th instar nymphs of *D. citri* were placed on filter paper located in the base of a 60 mm diameter Petri dishes.

Five different dishes were treated, each with a different conidial concentration, for a total of 30 Petri dishes for the six isolates. In addition, a different set of six Petri dishes were treated only with 0.03% Tween 80 solution as a control treatment, one Petri dish for each isolate. This represented 36 Petri dishes per occasion (replicate). For each conidial concentration, 1.2 mL of the suspension (or 0.03% Tween 80 for the control) was sprayed. For each isolate the control treatment was sprayed first followed by the lowest concentration and so on ending with the highest concentration. Between each concentration of the same isolate, the nozzle and the tower were cleaned by spraying 5 mL of 70% ethanol, followed by two washes of 5 mL of sterile distilled water.

Between isolates, 5 ml of 5% sodium hypochlorite were sprayed between the ethanol and the distilled water. Before each spraying, any liquid within the tower was removed using clean paper towels.

After treatment nymphs were transferred to leaflets of *M. paniculata* using a fine camel hair brush; nymphs from each replicate group were placed on a different leaflet. The petiole of the leaflet was inserted into a 1.5 mL Eppendorf tube containing sterile tap water to ensure the leaflet remained turgid during incubation. Each leaflet bearing nymphs was placed individually inside a 200 mL cylindrical plastic container. On one side of the container, a 3 cm diameter hole was made and covered with insect-proof

mesh to allow ventilation. On the other side of the container, a 1.3 cm diameter hole was made.

The 1.5 mL Eppendorf tube containing the *M. paniculata* leaflet was inserted through this hole, leaving the leaflet inside the plastic container in a horizontal position, and the tip of the Eppendorf tube outside the plastic container.

A paper towel moistened with 0.5 mL of sterile distilled water was placed inside the plastic container to maintain a relative humidity of approximately 60% within. All plastic containers were incubated at 25 °C in a light regime of 12:12 for seven days. Each day, *D. citri* mortality was recorded. Dead nymphs were incubated on sterile moistened filter paper in 60 mm diameter Petri dishes; any observed sporulation confirmed fungal infection as the cause of mortality. The experiment was conducted using a completely randomized design where all treatments were applied the same day and the complete experiment was replicated on three separate occasions (36 × 3 replicates = 108 Petri dishes).

Bioassays of adult *D. citri* and adults of the parasitoid *T. radiata* were conducted the same as the nymph assays described above with the following exceptions. Prior to inoculation, all groups of adult *D. citri* and *T. radiata* were exposed to 2 °C for 3 min to reduce their mobility during spraying. Inoculated adults were transferred to clean leaflets of *M. paniculata* as described for nymphs but the leaflets were transferred to a different type of transparent cylindrical plastic containers (5 cm diameter x 3 cm high) that had a hermetically sealed lid. The container had a 3 cm diameter hole covered with inset-proof mesh to allow ventilation.

A paper towel moistened with 5 mL of sterile distilled water was placed in the base of each container to produce a relative humidity of approximately 60%. All plastic containers were incubated at 25 °C in a 12:12 light regime for seven days for *T. radiata* and 15 days for adult *D. citri*. Confirmation of fungal infection was made in the same way as described for *D. citri* nymphs above. Adult *T. radiata* were provided with a drop of honey every day as food.

These experiments were both conducted using a completely randomized design where all treatments were made on the same day and the complete experiment was replicated on three separate occasions for the adult *D. citri* (36 × 3 replicates = 108 Petri dishes), and four separate occasions for *T. radiata* (36 × 4 replicates = 144 Petri dishes).

2.2.4 Statistical analyses

Statistical analyses for the two developmental stages of *D. citri* and the parasitoid were similar.

Mortality data were analysed using a generalized linear model (GLM) with binomial error and probit link, where the number of infected insects were assumed to follow a binomial distribution, with sample sizes equal to the number of insects tested. To compare amongst isolates, firstly a single line was fitted to data from all isolates. Secondly, intercepts were allowed to vary amongst isolates and thirdly, slopes were allowed to vary. Concentrations causing 50% infection (LC_{50}) were estimated from best fit models and confidence intervals (CI) for each LC_{50} were calculated according to Fieller's theorem (**Fieller, 1944**). In each experiment (replicate), a control treatment was included for each isolate tested.

Control mortalities had an average of 9.4%, 3% and 18.5% for *D. citri* nymphs, adults and *T. radiata* respectively. Mortalities in all fungal treatments were adjusted accordingly using the Henderson-Tilton formula (**Henderson and Tilton, 1955**). None of the dead control insects sporulated confirming no cross contamination between fungal and control treatments had occurred. All the statistical analyses were done using the statistical package GenStat v. 8.0 (**Payne et al., 2005**).

2.3 Results

2.3.1 Virulence of entomopathogenic fungi against *D. citri* nymphs and adults

For *D. citri* nymphs there were significant differences amongst intercepts ($F_{5, 78}$= 2.75, P=0.024), but not amongst slopes ($F_{5, 78}$= 1.90, P=0.104) for the six isolates evaluated. The differences among intercepts suggested a difference in virulence for the isolates tested against nymphs of *D. citri*.

The smallest LC_{50} value (4.25×10^6 conidial mL^{-1}) was for *M. anisopliae* isolate Ma129 and the largest LC_{50} value (1.56×10^{10} conidia mL^{-1}) was for the *I. fumosorosea* isolate I2 (Table 2.2).

For *D. citri* adults there were significant differences amongst both intercepts ($F_{5, 78}$= 4.21, P=0.002) and slopes ($F_{5, 78}$= 2.95, P=0.017) for the six isolates evaluated suggesting significant differences in virulence amongst the isolates tested. The smallest LC_{50} value (7.92×10^6 conidial mL^{-1}) was for *B. bassiana* isolate B1 and the largest LC_{50} value (3.31×10^{11} conidia mL^{-1}) was for *I. fumosorosea* isolate PAE (Table 2.2).

2.3.2 Virulence of entomopathogenic fungi against *T. radiata* adults

For *T. radiata* adults there were significant differences amongst intercepts ($F_{5, 78}$= 3.31, P=0.009), but not amongst slopes ($F_{5, 78}$= 0.22, P=0.954) for the six isolates evaluated.

The differences among intercepts suggested a difference in virulence for the isolates tested against adults of the parasitoid. The smallest LC_{50} value (1.49×10^8 conidial mL^1) was for *B. bassiana* isolate B1 and the largest LC_{50} value (3.67×10^{13} conidia mL^{-1}) was for the *I. fumosorosea* isolate I2 (Table 2.2).

2.4 Discussion

Nymphs of *D. citri* were susceptible to all isolates evaluated; overall, the *M. anisopliae* isolates Ma129 and Ma65 had the lowest LC_{50} values, and the two *I. fumosorosea* isolates (Pae and I2) the highest LC_{50} values (Table 2.2). The trend was the same for adults, except that the *B. bassiana* isolate B1 was the most virulent followed by *M. anisopliae* isolate Ma129. Overall, both developmental stages of *D. citri* were least susceptible to the two *I. fumosorosea* isolates. Isolates Ma65 (*M. anisopliae*) and B1 (*B. bassiana*) had been evaluated previously against *D. citri* adults when 97 and 85% infection was achieved for each isolate respectively at a conidial concentration of 1×10^8 conidia mL^{-1} (**Orduño-Cruz et al., 2015a**). In contrast, we achieved 60 and 73 % mortality with these two isolates at the same conidial concentration (data not shown); we believe these differences were due to the inoculation method used. **Orduño-Cruz et** al., (**2015 a**) inoculated *D. citri* adults by depositing 0.3 µL of the conidial suspension on to the abdomen of each insect and we sprayed conidial suspensions over the experimental dish, therefore it is likely that a different number of conidia reached the insects resulting in different mortality rates. For this reason, and because we are reporting dose-response assays, our results cannot be directly compared to the results reported by **Orduño-Cruz et al., (2015a)**.

Table 2.2. Virulence of the six fungal isolates evaluated against nymphs and adults of *D. citri*, and adults of *T. radiata*. LC_{50} (conidia mL^{-1}) ± 95% confidence interval (CI). RC=Regression coefficient (slope).

Isolate	LC_{50} (95% CI)	Intercept (SE)	RC (SE)
	D. citri nymphs		
Ma129	4.25×10^{6} (3.48×10^{4} - 1.99×10^{9})	-1.012 (0.30)	0.152 (0.04)
Ma65	6.20×10^{6} (5.59×10^{4} - 3.75×10^{9})	-1.05 (0.30)	""
B1	8.56×10^{6} (8.28×10^{4} – 6.52×10^{9})	-1.037 (0.30)	""
B3	1.73×10^{9} (1.44×10^{7} - 2.12×10^{14})	-1.55 (0.31)	""
Pae	2.77×10^{9} (2.12×10^{7} – 5.75×10^{14})	-1.41 (0.30)	""
I2	1.56×10^{10} (8.13×10^{7} – 2.38×10^{16})	-1.44 (0.30)	""
	D. citri adults		
B1	7.92×10^{6} (1.15×10^{5} - 2.83×10^{9})	-2.896 (0.61)	0.456 (0.09)
Ma129	5.27×10^{7} (8.83×10^{5} - 7.48×10^{10})	-0.939 (0.51)	0.113 (0.08)
B3	4.33×10^{9} (4.15×10^{7} - 3.63×10^{14})	-1.069 (0.78)	0.192 (0.12)
Ma65	1.39×10^{10} (1.02×10^{8} - 3.86×10^{15})	-0.853 (0.68)	0.040 (0.11)
I2	6.17×10^{10} (3.09×10^{8} - 8.20×10^{16})	-1.270 (0.51)	0.075 (0.08)
Pae	3.31×10^{11} (1.03×10^{9} - 2.70×10^{18})	-0.749 (0.35)	0.007 (0.05)
	T. radiata adults		
B1	1.49×10^{8} (2.40×10^{6} – 8.62×10^{11})	-1.468 (0.35)	0.1796 (0.05)
B3	2.60×10^{8} (3.94×10^{6} – 2.60×10^{12})	-1.511 (0.35)	""
Ma129	4.82×10^{9} (4.26×10^{7} – 1.15×10^{15})	-1.739 (0.35)	""
Ma65	1.97×10^{10} (1.22×10^{8} – 2.40×10^{16})	-1.849 (0.36)	""
Pae	3.82×10^{10} (1.97×10^{8} - 1.01×10^{17})	-1.900 (0.36)	""
I2	3.67×10^{13} (1.89×10^{10} – 4.69×10^{23})	-2.436 (0.39)	""

It has been suggested that entomopathogenic fungi may be more virulent when applied against individuals of the same host species from which they were originally isolated (**Goettel, 1994; Cabanillas and Jones, 2009**). In our experiments, isolate B1 showed this relationship as it was more virulent against adult *D. citri*, which was the host from which it was isolated (**Orduño-Cruz et al., 2015b**).

However, this isolate (B1), was not the most virulent against *D. citri* nymphs (Table 2.2), suggesting that virulence was not only dependent on the host species but also the developmental stage. Although this confirms previous reports (**Petersen-Silva et al., 2015**), our results need further confirmation by evaluating a greater number of isolates and hosts.

From a practical point of view, our data and previously reported data (**e.g. Avery et al., 2011; Stauderman et al., 2012; Gandarilla-Pacheco et al., 2013; Cortez-Madrigal, et al., 2014**) showed that entomopathogenic fungi have great potential for biological control of *D. citri*. Based on the LC_{50} values estimated for *D. citri*, nymphs were more susceptible than adults, which suggests that control efforts should focus on this developmental stage. Furthermore, nymphs lack mobility and are gregarious in young leaflets making them a better target for biological control (**Fernández and Miranda, 2005**). However, we cannot ignore the importance of adult *D. citri* which, being a very active stage, are more effective at disseminating the bacterium, HLB.

Therefore, using isolates with the potential to infect both developmental stages is also very important. Recently, **Orduño-Cruz et al., (2016)** using some of the isolates studied here (Ma65 and B1) reported that *D. citri* carrying the bacterium was more susceptible to fungal infection compared to *D. citri* without the bacterium, representing a possibility

to achieve greater mortalities in adult *D. citri* in comparison with the results we are presenting here.

The ability to infect the target pest insect is just one biological attribute of entomopathogenic fungi that requires evaluation before field release.

One of the most important attributes to be considered is their impact on non-target organisms (**Khan et al., 2012**). In this respect, the parasitoid *T. radiata* is an important biological control agent of *D. citri* (**Chen and Stansly, 2014**) and it is very likely that both organisms (fungi and parasitoid) will interact with each other once released in the field. Therefore, it is important to study the potential outcomes of these interactions in order to use them both efficiently to reduce *D. citri* populations in the field. As a first step to achieving this, we studied the relative susceptibility of *T. radiata* and *D. citri* to the same fungal isolates. Our results showed that all isolates infected the parasitoids, and the most virulent isolates against *T. radiata* were the *B. bassiana* isolates B1 and B3, followed by *M. anisopliae* and finally the *I. fumosorosea* isolates as the least virulent (Table 2.2). However, the parasitoids were less susceptible than *D. citri*; for example, isolate Ma129 was 1000 times more virulent against *D. citri* nymphs compared with the parasitoids (Table 2.2). This suggests that if this isolate were applied in the field targeting the nymphs, it would be unlikely that this would have a negative impact on the parasitoid.

However, the infection results obtained after insects were inoculated in the laboratory (physiological hosts) are not necessarily reproducible in the field (ecological hosts) (**Vega et al., 2012**); therefore, our hypothesis requires experimental confirmation in the field.

Despite the fact that all fungal isolates infected *D. citri* and its parasitoid, which may cause a conflict when selecting isolates and field doses, we consider that the combined use of both biological control agents could still be possible, in addition to the differences in virulence between *D. citri* and *T. radiata*, some temporal separation between them could be achieved, to avoid parasitoid infection.

Furthermore, as **Goettel (1994)** states, although some insects can be infected in the laboratory, the lack of reports of natural infection in the field suggests that field infection is unlikely. We are currently assessing these interactions between the two biological control agents in more detail within *D. citri* populations using isolates MA129 and B1, which were the most virulent against both developmental stages of *D. citri*.

In summary, our data suggest that the six fungal isolates tested here could infect both *D. citri* and *T. radiata.* We propose isolates Ma129 and B1 as strong candidates for the control of *D. citri*. However, their use in combination with *T. radiata* although potentially possible, still requires further research to design a biological control programme that makes the most of both agents. For example studies on the effect of a time separation between releases, on the order of inoculation and on the ability of the parasitoid to discriminate between infected and healthy hosts.

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Capítulo 3. Interactions between entomopathogenic fungi and *Tamarixia radiata* (Hymenoptera: Eulophidae) in *Diaphorina citri* (Hemiptera: Liviidae) populations

Abstract

Biological control of *Diaphorina citri* (Kuwayama) using the parasitoid, *Tamarixia radiata* (Waterston), and entomopathogenic fungi has been attempted, but there have been no studies on their potential interactions when used together. We studied the effect of prior residency time on the outcomes of interactions between *T. radiata* and the fungal pathogens *Beauveria bassiana* and *Metarhizium anisopliae* in *D. citri* nymphs. Firstly, nymphs that had been infected with fungi for different times (0, 24 and 72 h) were exposed to the parasitoid. Secondly, nymphs carrying parasitoids at different developmental stages (eggs, larvae or pupae) were exposed to fungal inoculation. The greatest proportion of fungus-infected nymphs occurred when they were inoculated 72 h prior to parasitoid exposure. The lowest parasitism rate occurred in nymphs that had been infected by fungi 72 h prior to parasitoid exposure. The number of nymphs used for host-feeding by the parasitoid was similar, regardless of how advanced fungal infection was. When fungal inoculations were made to nymphs carrying different developmental stages of the parasitoid, the greatest proportion becoming infected occurred in nymphs carrying eggs.

The overall longevity of adult parasitoids emerging from control and fungal-infected treatments was similar; however, the longevity of adult parasitoids emerging from nymphs that were inoculated when they were carrying parasitoid larvae were the lowest,

with a greater effect for the *M. anisopliae* isolate. The ecological importance and practical recommendations derived from our results are discussed.

Keywords: Multitrophic interactions; *Beauveria bassiana*, *Metarhzium anisopliae*, parasitism, infection, host feeding.

3.1 Introduction

Diaphorina citri (Kuwayama) is one of the most important pests attacking citrus crops worldwide (**Bové, 2006**). Direct damage is caused by feeding when *D. citri* extracts the sap thereby weakening the plant and at the same time injects toxins that drastically reduce bud growth and deform leaves (**Myevo Nkankeo et al., 2011**). However, the most significant damage caused by *D. citri* is as a result of transmission of the devastating citrus disease, Huanglongbing (HLB) (**EPPO/CABI, 1996**). This disease is caused by the bacterium *Candidatus* liberibacter *asiaticus* (**Michaud, 2004; Bové, 2006**). Although chemical insecticides are currently the most common control strategy, use of natural enemies such as the parasitoid, *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae), has also been attempted (**Hall, 2008**). Furthermore, the potential of entomopathogenic fungi such as *Isaria fumosorosea* Wize, *Beauveria bassiana* s.l. (Bals) Vuill., *Metarhizium anisopliae* s.l. Metch. Sorokin and *Hirsutella citriformis* Speare has also been evaluated under laboratory conditions (**Hall et al., 2013; Orduño-Cruz et al., 2015a, b**).

However, before large-scale experimentation with these parasitoids and pathogens, it is important to quantify any potential positive and/ or negative interactions that may occur between them. This would facilitate the design of more effective control strategies for

using these natural enemies, either singly or in combination, within an integrated pest management system.

The combined use of pathogens and arthropod natural enemies for the biological control of pests has been suggested previously (**e.g. Roy and Pell 2000; Nielsen et al. 2005**); however, it is a prerequisite to have as much information as possible about the ecology of these interactions, before combinations of natural enemies can be used efficiently. When more than one natural enemy exploits the same host, an interaction between both organisms is likely to occur, which can result in different outcomes with respect to the host population (**Furlong and Pell 2005**). For example, when using entompathogenic fungi that are considered as generalists (such as *B. bassiana* or *M. anisopliae*), there is a high risk that the insect natural enemy may become infected as well as the target pest (**Roy and Pell 2000**). **Tamayo-Mejía et al., (2015)** found that, during oviposition, the ectoparasitoid *Tamarixia triozae* Burks could not distinguish between *B. bassiana*-infected and uninfected nymphs of its host *Bactericera cockerelli* Sulc.; this resulted in a reduced longevity of the parasitoid after it had been in contact with the infected host. However, when fungal inoculations were made against *B. cockerelli* nymphs carrying different developmental stages of the parasitoid, older stages of the parasitoid were less susceptible to infection (**Tamayo-Mejía et al., 2016**). This suggests that the combined used of both natural enemies is possible, but that a temporal separation in release time of each organism is required.

In Mexico, *T. radiata* has been released in the field for control of *D. citri* since 2010 (**Arredondo-Bernal et al., 2010**). While field applications of *M. anisopliae* s.l. and *I. fumosorosea* have been made in citrus production areas in Mexico, to date there are no

formal reports or publications concerning the results of these applications. For this reason, interactions between *T. radiata* and entomopathogenic fungi are likely to have happened in Mexican citrus production areas; this makes studies that provide an understanding of potential interactions even more important as they may shed light on unexpected control improvements/ failures and underpin the development of more efficient strategies for the combined use of these two natural enemies.

Recently, we have studied the susceptibility of *T. radiata* adults, and the nymphs and adults of *D. citri*, to six isolates of entomopathogenic fungi, two isolates each of the species *B. bassiana* s.l., *M. anisopliae* s.s. and *I. fumosorosea* (**Ibarra-Cortés et al. paper submitted**). Results demonstrated that the parasitoid was much less susceptible to fungi than *D. citri*, suggesting that the combined use of fungi and parasitoids was possible. Using this information, we were able to select a reduced number of isolates for use in interaction experiments. Here we present data from these experiments were the effect of order of arrival, parasitoid developmental stage, and stage of infection were studied in *D. citri* nymphs.

3.2 Materials and methods

3.2.1 Rearing *Diaphorina citri* and *Tamarixa radiata*

Nymphs and adults of *D. citri* and *T. radiata* were provided by Centro Nacional de Referencia de Control Biológico (CNRCB).

All insect cultures were maintained in greenhouses at the CNRCB premises. The *D. citri* colony was maintained on *Murraya paniculata* (L.) Jack (Sapindales: Rutaceae) plants. In a separate greenhouse, 3rd, 4th and 5th instar of *D. citri* were used to produce *T. radiata* adults.

3.2.2 Entomopathogenic fungal isolates and production of inoculum

We used monosporic reisolations of both *B. bassiana* isolate B1, originally from *D. citri* (**Orduño-Cruz et al., 2015a**) and *M. anisopliae* isolate Ma129, originally from the mite *Tetranychus urticae* Koch (Trombidiformes: Tetranychidae). The isolates had been evaluated previously against *D. citri* and *Maconellicoccus hirsutus* Green by **Ibarra-Cortés et al., (2013)**; **Orduño-Cruz et al., (2015b)**, and are currently held in the culture collection of entomopathogenic fungi at the Insect Pathology Laboratory, Colegio de Postgraduados, Mexico. Isolates were retrieved from -80 °C storage and grown on Sabouraud Dextrose Agar (SDA) plates at 25 °C in complete darkness for 15 days prior to experimentation. Isolates were not subcultured more than three times following retrieval from storage at -80 °C. After incubation, conidia were collected from SDA plates using a sterile scalpel and deposited into a 50 mL centrifuge tube containing 20 mL of 0.03% sterile Tween 80 solution. All these procedures were done under sterile conditions in a laminar flow cabinet.

Centrifuge tubes containing fungal material in Tween 80 were vortexed for five minutes at maximum speed and then filtered into a new centrifuge tube using a sterile cloth, to remove mycelium from the conidial suspension. A 1 µL aliquot of the resulting conidial suspension was deposited into a 1.5 mL Eppendorf type tube containing 999 µL of 0.03% Tween 80 solution.

This suspension was used to estimate the conidial concentration of the stock suspension using a Neubauer haemocytometer; the concentration was then adjusted to 8.5×10^6 and 4.2×10^6 conidia mL^{-1} for *B. bassiana* and *M. anisopliae*, respectively.

These concentrations represent the LC_{50} values for these isolates estimated against *D. citri* nymphs by **Ibarra-Cortés et al., (paper submitted).**

3.2.3 Experiment to determine parasitism by *T. radiata* of *D. citri* nymphs infected by fungal pathogens for different periods of time

The methodology for inoculating *D. citri* nymphs with fungal suspensions was the same for both isolates. Fourth instar nymphs of *D. citri* were placed on to the bases of 60 mm diameter Petri dishes containing damp filter paper. Inoculations were made using a spray tower (glass cylinder of 51 cm diameter and 20 cm height) fitted with a cone spray nozzle (Spraying Systems Co. Wheaton, IL, USA) attached to an air compressor at 20 psi. Each group of 30 nymphs (in one Petri dish) were inoculated with 5 mL of the conidial concentration selected for each isolate and a number of groups sprayed to provide replicate groups. After inoculation, each group of nymphs was transferred to a *M. paniculata* leaflet using a fine brush.

The petiole of each leaflet was inserted in to a 1.5 mL Eppendorf type tube filled with tap water to ensure the leaflets remained turgid. The boundary between the Eppendorf tube and the leaflet was sealed with parafilm to prevent evaporation.

Each leaflet containing the nymphs was then placed individually inside a cylindrical lidded 200 mL plastic container through a 1.3 cm diameter hole made in the side of the plastic container, leaving the leaflet inside the container in a horizontal position, and the tip of the Eppendorf tube outside the container; on the other side of the container a lateral 5 cm diameter hole was made and covered with mesh for ventilation. A paper towel (5 x 5 cm) moistened with 0.5 mL of sterile distilled water was placed inside the

plastic container to ensure a relative humidity of approximately 60% was maintained inside.

Using the procedure described above, we produced, on the day of the experiment, groups of nymphs that had been inoculated with fungi 0, 24 and 72 hours (treatments) previously. Two adult two-day-old female *T. radiata* were introduced in to each replicate container harbouring groups of nymphs at different stages of infection, and allowed to forage for 24 hours; parasitoids were then removed using a mouth aspirator. Simultaneously, three control treatments were also set up. The first control group consisted of nymphs that had been inoculated with 5 mL of 0.03% Tween 80 solution only (no fungus) and had not been foraged upon by parasitoids for 24 hours. The second control group consisted of nymphs inoculated with one or other of the two fungal isolates as described above, but that had not been foraged upon by parasitoids for 24 hours.

The third control group consisted of nymphs that had been inoculated with 5 mL of 0.03% Tween 80 only (no fungus) but had been foraged upon by parasitoids for 24 hours. All containers were incubated at 25±2°C, 60% H.R and in a 12:12 light: dark regime.

The complete experiment was assessed only once after the containers had been incubated for seven days. Three sets of data were collected: the number of nymphs infected, parasitized or used as food by the parasitoid (i.e. host-feeding behaviour). To determine the number of nymphs infected, dead nymphs were placed in 5 cm diameter Petri dishes lined with damp filter paper and incubated for a further five days under the

same conditions to encourage sporulation and confirm (or otherwise) whether the mortality was due to infection. Simultaneously, the number of parasitized nymphs, or nymphs used as a food source by the parasitoid (nymphs without haemolymph, without dorsal symmetry and heavily attached to the leaf) were recorded. To assess parasitism nymphs were incubated for a further 10 days and the emergence of adult parasitoids recorded as representative of the number of nymphs parasitized. In addition, each newly emerged adult parasitoid was individually placed in to a transparent plastic vial (2.7 cm diameter x 5 cm height) with a lid. To allow ventilation, approximately 10 holes were made in the lid with a red-hot entomological needle. Parasitoids were fed with a drop of honey placed in the inner part of the lid, and incubated under the same conditions until the last one died. This allowed us to record the longevity of the adult parasitoids that emerged from nymphs in each treatment.

3.2.4 Experimental design and statistical analyses

The experiment was done using a completely randomized design. All treatments were established the same day, and the complete experiment was repeated on four different occasions.

The first control treatment (with no fungus or parasitoid applied) was excluded from the analysis as no mortality was recorded. Data were analysed using logistic regression, assuming a binomial distribution and the number of nymphs responding in each treatment as a proportion of the total number of nymphs tested.

When analysing infection rates: firstly, the fungus-only treatments (second control) were compared to the combined fungus and parasitoid treatments; secondly, within the

fungus-only treatments, a nested contrast defined by isolate and the different infection times was assessed and their interactions evaluated; thirdly, within the combined treatments (fungus and parasitoid), the proportion of infection was analysed using the same nested contrast defined by isolate and the different infection times and their interaction evaluated.

When analysing the proportions of *D. citri* nymphs parasitized: firstly, data from parasitoid-only treatments (third control) were applied was compared to treatments were both fungus and parasitoids were applied; secondly, within treatments where only parasitoids were released, the proportion of parasitism amongst the three times (0, 24 and 72 h after initiation) after which the parasitoids were applied, but without any fungal inoculation were compared; thirdly, the parasitism data from treatments where both fungi and parasitoids were applied, were analysed using the same nested contrasts structure as described for the infection data, which was defined by isolate, infection times and their interaction. The same method was used when the proportions of nymphs used for host-feeding were analysed.

Analysis of the longevity data (number of days that each adult parasitoid survived) was compared amongst treatments using ANOVA with an unbalanced design; this was due to the fact that different numbers of adult parasitoids emerged from nymphs in the different treatments (each adult representing a repetition). A similar analysis structure as described above was incorporated into the analysis, comparing first treatments were only parasitoids were applied against all fungal and parasitoids treatments combined. Within the fungi and parasitoids treatments, the longevity of the parasitoids was analysed using a nested contrast for isolate and infection times followed by their

interaction. All analyses were done using the software GenStat v. 8.0 (Payne et al., 2005).

3.2.5 Experiment to determine the effect of inoculation with entomopathogenic fungi on juvenile stages of *T. radiata*

Groups of 30 4th instar *D. citri* nymphs, each parasitized and carrying either eggs (two days after oviposition), larvae (four days after oviposition) or pupae (ten days after oviposition) of *T. radiata*, were each inoculated with one or other of the two isolates using the same conidial concentrations and method described for the previous experiment.

After fungal inoculations, all groups of treated nymphs carrying each of the different developmental stages of *T. radiata*, were placed on to *M. paniculata* leaflets as described previously (except nymphs carrying pupae of the parasitoid) and incubated under the same conditions.

Inoculated nymphs carrying pupae of the parasitoid were placed in 50 mm Petri dishes with damp filter paper in the base and incubated under the same conditions. Simultaneously, three control groups were set up. The first control group was nymphs that had not been parasitized and were then inoculated with 0.03% Tween 80 only (no fungus). The second control group consisted of groups of nymphs, each group carrying either eggs, larvae or pupae of the parasitoid, that were then inoculated with with 5 mL of 0.03% Tween 80 only (no fungus), representing a control treatment for each developmental stage of the parasitoid. The final control group consisted of two groups

of unparasitized nymphs that were inoculated with one or other of the two fungal isolates.

3.2.6 Infection of all developmental stages of *T. radiata* as well as un-parasitized nymphs inoculated with the two isolates were recorded once, seven days after initiation of the experiment.

Dead insects were placed in to 5 cm diameter Petri dishes on damp filter paper, and incubated under the same conditions for a further five days to encourage sporulation and confirm infection. Parasitoid emergence was recorded every 24 hours starting six days after the experiment was initiated. All adult parasitoids that emerged were handled and maintained individually as described for the previous experiment. Assessments of survival were made every 24 hours until the last parasitoid died.

3.2.7 Experimental design and statistical analyses

As with the previous experiment, a completely randomized design was used where all treatments were set up on the same day, and the complete experiment was repeated on four different occasions. The proportion of adult parasitoids emerging from all treatments was analysed using logistic regression. Firstly, the proportion of adult parasitoids emerging from treatments with no fungal inoculation (second control, combining the three developmental stages) was compared to the proportion of parasitoids emerging in treatments where fungi had been applied (combining the three developmental stages). Within the parasitoid-only treatments, the effect of the three developmental stages of the parasitoids on the proportion that emerged was compared. Then, using data from the parasitoid plus fungi treatments, a nested contrast using a factorial set of treatments defined by isolate and developmental stage of the parasitoid

and their interaction was made. The fungus-infected proportion of *D. citri* nymphs carrying the three developmental stages of the parasitoids were analysed using logistic regression. Firstly, the proportion of *D. citri* nymphs infected in treatments where they were carrying the different developmental stages of the parasitoid were compared with treatments where they were not parasitized (third control). Within the non-parasitized nymphs, the effect of isolate was tested. Using data from *D. citri* nymphs carrying the three developmental stages of the parasitoid, a nested contrast for the factorial set of treatments defined by isolate and developmental stage of the parasitoid were estimated and tested.

As the numbers of adults that emerged from control and fungal treatments were not similar, the longevity of the emerged adults was analysed using ANOVA for unbalanced designs. No transformation of data was required. First, a comparison between the longevity of parasitoids emerging from treatments that had been inoculated with fungi was compared with treatments without fungal inoculation (second control). The longevity of parasitoids emerging from treatments without fungal inoculation were compared, followed by a comparison of the longevity of parasitoids that emerged from nymphs where they had received fungal inoculum at different developmental stages. Using data from treatments where both fungi and parasitoids were applied, nested contrasts for the factorial set of treatments and their interactions defined by isolate and developmental stage of the parasitoid was assessed.

3.3 Results

3.3.1 Parasitism by *T. radiata* of *D. citri* nymphs infected by fungal pathogens for different periods of time

The overall proportion of infected *D. citri* nymphs was greater in the treatments where only fungi were inoculated (0.38, C.I. [confidence intervals] = 0.441-0.340) compared with the treatments where fungi and parasitoids were both applied (0.27, C.I.= 0.327-0.231) ($F_{1, 36}$=8.95, P=0.005). In treatments where fungi and parasitoids were combined, no differences were found in the proportion of *D. citri* nymphs infected between the two isolates ($F_{1, 36}$= 0.66, P=0.420); however, significant differences were found in the proportion of *D. citri* nymphs infected amongst the three different infection times at which the parasitoid was applied ($F_{2, 36}$= 7.51, P=0.002).

More nymphs of *D. citri* became infected when parasitoids were applied after the fungi had been inoculated 72 h previously (Fig. 3.1), and this was regardless the isolate used ($F_{2, 36}$= 0.11, P=0.896).

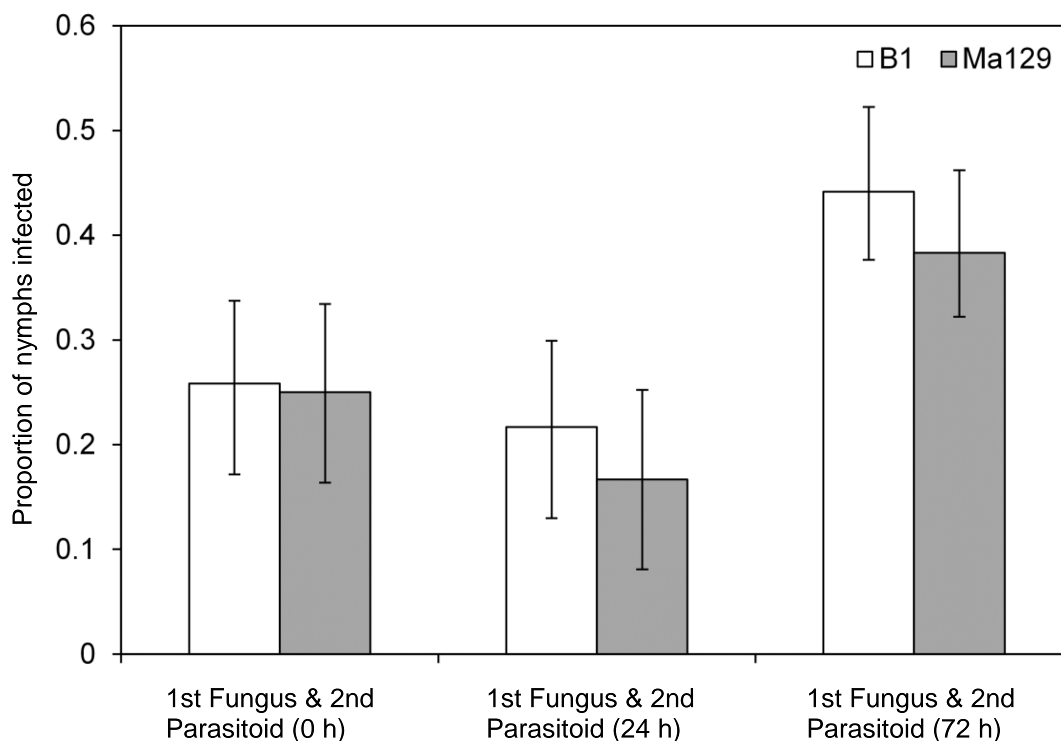

Figure 3.1: Proportion of *D. citri* nymphs infected with *B. bassiana* or *M. anisopliae* when they were first inoculated with fungus and then placed in contact with the parasitoid, *T. radiata*, 0 (Fungi 0 h), 24 (Fungi 24 h) or 72 (Fungi 72 h) hours after fungal inoculation. Error bars represent 95% confidence intervals back-transformed from the logistic scale.

The proportion of nymphs parasitized by *T. radiata* was greater in the treatments where no fungi were inoculated (0.43, C.I.= 0.475-0.400) compared with the treatments where fungi and parasitoids were both applied (0.15, C.I.= 0.189-0.128) (χ_1^2=117.16, P<0.001). When the parasitoids were applied to nymphs at 0, 24 and 72 h after the experiment was initiated (without any fungal inoculation), no differences were found amongst the proportion of of *D. citri* nymphs that became parasitized (χ_2^2= 0.8507, P=0.654); the mean proportion of nymphs parasitized was 0.41. In treatments where fungi and

parasitoids were both applied, the proportions of *D. citri* nymphs that were parasitized were not different between the two isolates tested (χ^2_1=0.2781, P=0.870) (Fig. 3.2A). However, the proportion of *D. citri* nymphs that were parasitized was dependent on whether the parasitoids were applied 0, 24 or 72 h after fungal inoculation (χ^2_2=40.592, P<0.001), and this result was regardless of the isolate used (χ^2_2= 4.404, P=0.111). The greatest proportion of *D. citri* nymphs that were parasitized was achieved when parasitoids were applied 24 after fungal inoculation (Fig.3. 2A).

No differences were observed in the proportions of nymphs used for parasitoid host-feeding between treatments where the fungi and parasitoids were both applied and treatments where only the parasitoids were applied (χ^2_1= 0.471, P=0.492); the mean proportion of *D. citri* nymphs used for host-feeding was 0.31.

When only the parasitoids (no fungi) were applied to *D. citri* nymphs 0, 24 and 72 h after the experiment was initiated, no differences were found in the proportion of *D. citri* nymphs used for host-feeding amongst the three times (χ^2_2= 1.635, P=0.442); the mean proportion of *D. citri* nymphs used for host-feeding at the three times was 0.30.

In treatments where fungi and parasitoids were both applied, the proportion of *D. citri* nymphs used for host-feeding was similar between the two isolates (χ^2_1= 1.267, P=0.531) (Fig. 3.2B).

Significant differences were found in the proportions of *D. citri* nymphs used for host-feeding in treatments when the parasitoid was released 0, 24 or 72 hours after the fungi

were inoculated (χ^2_2= 9.20, P=0.010), and there was no interaction with the fungal isolate used (χ^2_2= 0.910, P=0.634). The smallest proportion of *D. citri* nymphs used for host-feeding occurred when parasitoids were applied 24 h after the fungi had been inoculated, compared with when they were released 0 and 72 hours after fungal inoculation (Fig. 3.2B).

The longevity of parasitoids that emerged as adults from treated nymphs was greater in treatments where only the parasitoids were applied, with an mean longevity of 23.09 (± 0.530) days compared with the longevity parasitoids emerging in treatments where fungi and parasitoids were both applied, which was 18.97 ± 0.906 days ($F_{1, 382}$=16.66, P<0.001). Within the parasitoid and fungi treatments, there was: no difference between isolates ($F_{1, 382}$= 0.02, P=0.879); no difference amongst the three times post-fungal inoculation that parasitoids were applied ($F_{2, 382}$= 0.12, P=0.890); and no interaction between these two factors ($F_{2, 382}$= 0.78, P=0.457).

3.3.2 Effect of inoculation with entomopathogenic fungi on *T. radiata* juvenile stages

The proportion of adult parasitoids that emerged was greater in treatments where only parasitoids were applied (0.73, C.I.= 0.783-0.690) compared with the treatments where fungi and parasitoids were both applied (0.43, C.I.= 0.490-0.374) ($F_{1, 39}$= 69.68, P<0.001). The proportion of adult parasitoids that emerged from treatments where only parasitoids were applied was similar amongst the treatments containing different initial developmental stages of the parasitoid ($F_{2, 39}$= 0.91, P=0.411); the mean proportion of parasitoids emerging was 0.73. In treatments where fungi and parasitoids were both

applied, there was no difference in parasitoid emergence between treatments using the two different fungal isolates ($F_{1, 39}$= 0.05, P=0.830) (Fig 3.3A). Within the fungi and parasitoids treatments, the parasitoid emergence was different amongst treatments where different developmental stages of the parasitoid were inoculated with fungi ($F_{2, 39}$= 31.45, P<0.001), regardless the isolate used ($F_{2, 39}$= 0.05, P=0.948). Greater parasitoid emergence was achieved when the parasitoids were inoculated with fungi as pupae (Fig. 3.3A).

The proportion of non-parasitized *D. citri* nymphs that became infected with fungi (0.52, C.I.= 0.570-0.470) was greater than the combined proportion of *D. citri* nymphs becoming infected when they were carrying the three developmental stages of the parasitoid (0.38, C.I.= 0.440-0.332) ($F_{1, 40}$= 10.80, P=0.002). There were no differences in the proportion of non-parasitized nymphs becoming infected between the two isolates tested ($F_{1, 40}$= 3.46, P=0.07).

Significant differences were found in the proportions of *D. citri* nymphs becoming infected when they were carrying the three developmental stages of the parasitoid ($F_{2, 40}$= 25.37, P<0.001); the highest proportion of infected *D. citri* nymphs was achieved when nymphs were carrying the egg of the parasitoid and the lowest proportion of infected *D. citri* nymphs was achieved when nymphs were carrying pupae of the parasitoid (Fig 3B). This result was similar for the two isolates ($F_{1, 40}$= 0.36, P=0.552) (Fig. 3.3B), and there was no interaction between these two factors ($F_{2, 40}$= 0.48, P=0.624).

The longevity of parasitoids that emerged as adults was similar in treatments where no fungi were inoculated (19.23 ± 0.661 days) compared with when fungi were inoculated, where the mean longevity was 18.51 (±0.903) days ($F_{1, 207}$= 0.54, P=0.463). Within treatments with no fungal inoculation, the longevity of the adult parasitoids that emerged were different amongst the different initial developmental stages of the parasitoid ($F_{2, 207}$= 6.75, P=0.001); greatest longevity was achieved by parasitoids that emerged from *D. citri* nymphs parasitized with pupae (Fig. 3.4A). Within the fungal-inoculated treatments, significant differences were found amongst the longevity of parasitoids that emerged from *D. citri* nymphs parasitized with the different developmental stages of *T. radiata* ($F_{2, 207}$= 6.76, P=0.001); the greatest longevity was achieved by parasitoids that emerged from *D. citri* nymphs carrying eggs when the fungi were inoculated (Fig. 3.4B).

However, there was a significant interaction with the isolate used ($F_{2, 207}$= 9.68, P<0.001); despite longevity being similar between parasitoids that emerged from *D. citri* nymphs carrying pupae inoculated with either of the the two isolates (Fig 3.4B), the longevity of parasitoids that emerged from *D. citri* nymphs carrying larvae when inoculated with the *M. anisopliae* isolate was greatly reduced compared with parasitoids that emerged from treatments inoculated with the *B. bassiana* isolate (Fig. 3.4B).

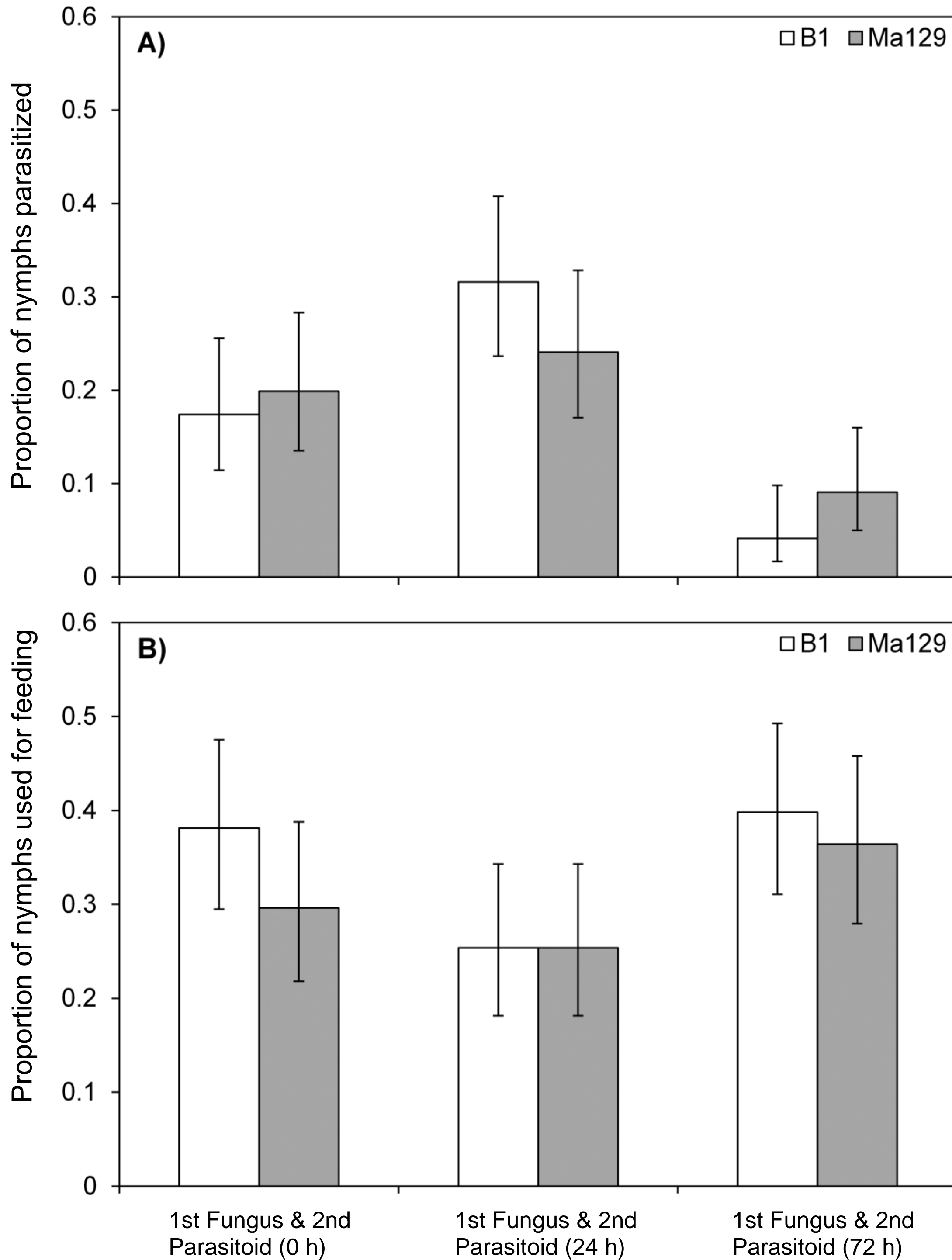

Figure 3.2: Proportion of *D. citri* nymphs parasitized (A) or used for host-feeding (B) by the parasitoid, *T. radiata*, when they had previously been infected by *B. bassiana* or *M. anisopliae* for 0, 24 or 72 hours. Error bars represent 95% confidence intervals back-transformed from the logistic scale.

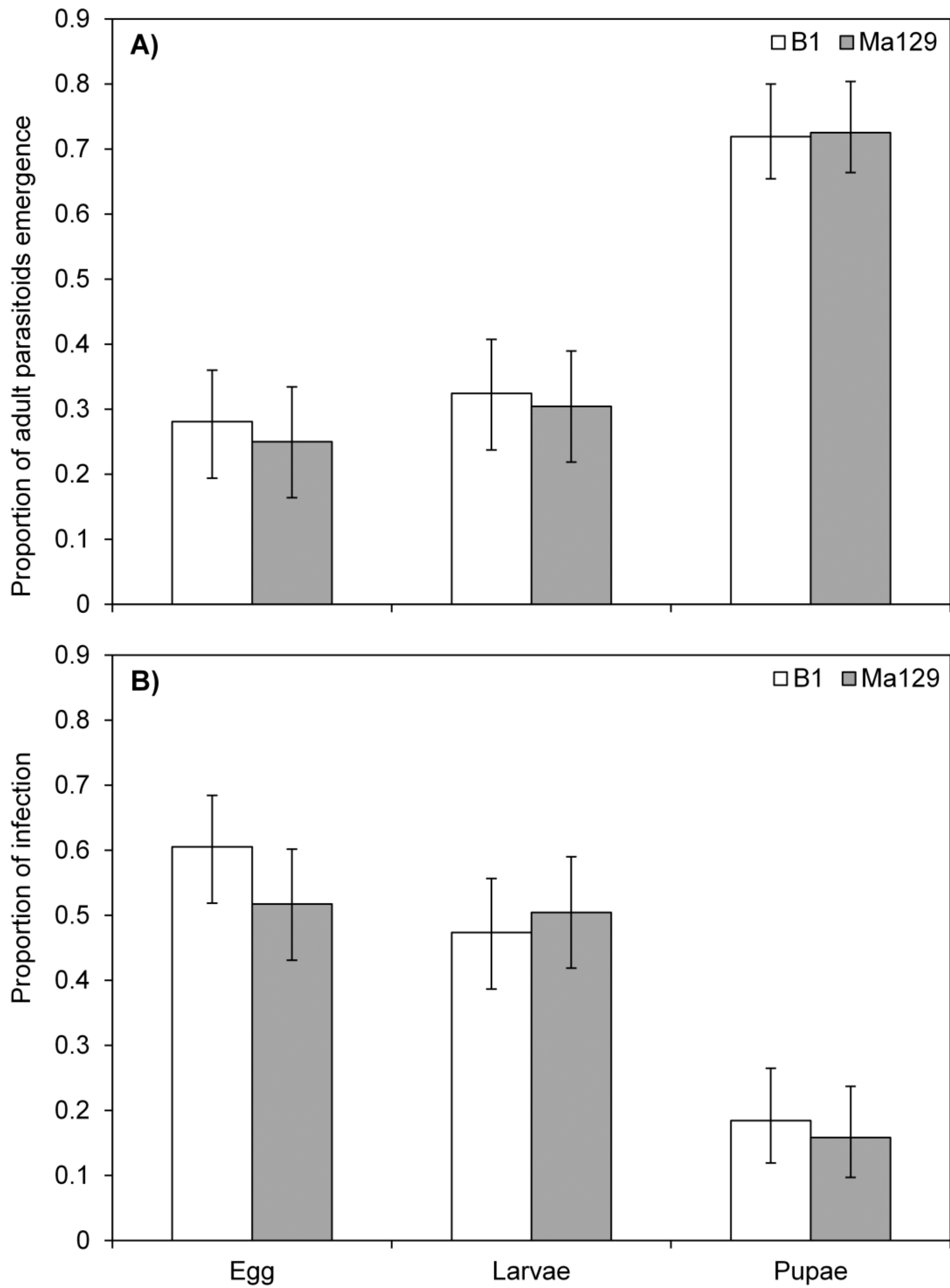

Figure 3.3: Proportion of adult parasitoids emerging (A), and proportion of *D. citri* nymphs becoming infected with *B. bassiana* or *M. anisopliae* when they were carrying eggs, larvae or pupae of the parasitoid, *T. radiata*, at the time of fungal inoculation (B). Error bars represent 95% confidence intervals back-transformed from the logistic scale.

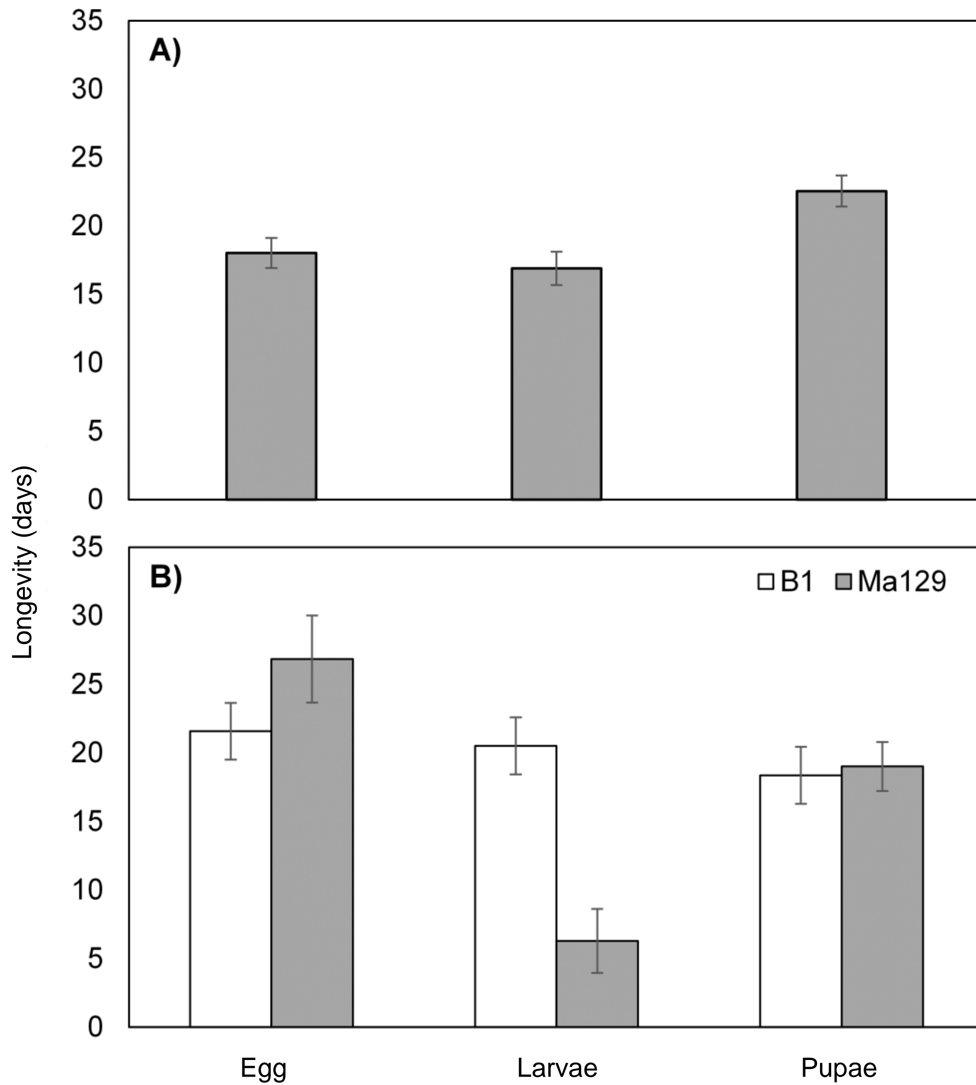

Figure 3.4: Longevity of adult *T. radiata* parasitoids emerging from non-fungus-inoculated *D. citri* nymphs (A) and *D. citri* nymphs inoculated with the *B. bassiana* or *M. anisopliae* isolate (B). All nymphs were initially carrying eggs, larvae or pupae of the parasitoid. Error bars represents 1 ± SEM).

3.4 Discussion

Biological control of pests is an important alternative to the use of chemical insecticides. However, basic studies are normally required for a biological control programme to be successfully implemented. For example, whether an entomopathogenic fungi or a parasitoid are to be released, the probability of interactions between these two organisms during competition to exploit the same host, are significant. There are many papers reporting the outcomes of such interactions; results vary from the parasitoid being practically non-susceptible to fungal infection compared with its host (**Dean et al., 2012; Rossoni et al., 2014**), to the parasitoid been highly susceptible to fungal infection where a temporal separation is recommended to avoid antagonistic interactions (**Aqueel and Leather 2013; Emami et al., 2013; Martins et al., 2014; Tamayo-Mejía et al., 2015, 2016**).

Overall, our results showed that *T. radiata* was susceptible to infection by *B. bassiana* (B1) and *M. anisopliae* (Ma129). No difference was found between the isolates in the majority of the experiments, suggesting that the difference in doses based on estimated LC_{50} values were effective. It is clear that prior residency in the host provided a competitive advantage to both natural enemies when using *D. citri* nymphs as a host. The advantage of prior residency has been reported previously in other systems (**de Roode et al., 2005; Lohr et al., 2010**), where it is assumed that an organism attempting to exploit a host that is already colonized by another organism is at a disadvantage. This was observed when fungal-inoculated nymphs were exposed to *T. radiata* after different infection times.

The greatest fungal infection was achieved when the parasitoids were introduced 72 hours after fungal inoculation took place, compared with the treatments where both were applied simultaneously (Fig. 3.1). Similarly, successful parasitism was greatly reduced when parasitoid oviposition occurred at the same time as fungal infection (Fig. 3.2A). *Tamarixia radiata* is a synovigenic parasitoid (**Jervis and Kidd 1986**), which means it can also use its host for feeding; interestingly, this behaviour was not affected by the period of time that the host had been infected by the fungus (Fig. 3.2B). We consider this attribute important because it suggests that *T. radiata* can distinguish healthy from fungus-infected *D. citri* nymphs, parasitizing only uninfected hosts and thereby ensuring progeny survival, but can feed on all nymphs regardless of infection level. Such behaviour could be driven by the urgent need of the parasitoid to obtain the necessary protein reserves required for development of their eggs (**Hoy et al., 2006**). If entomopathogenic fungi were to be released before the parasitoids, the fungi would be negatively affected because the parasitoid would subsequently feed on infected *D. citri* nymphs, reducing the likelihood of the fungi surviving to reproduce (sporulate) on their host. However, it is also possible that the parasitoid´s behaviour of feeding on infected hosts may increase the transmission of the pathogen within a host population, compensating for the reduction in fungal inoculum (**Roy and Pell 2000**).

In contrast, when *D. citri* nymphs carrying different developmental stages of the parasitoid were inoculated with fungi, the greatest adult emergence of parasitoids was obtained when the fungi were applied to *D. citri* nymphs carrying parasitoid pupae compared with *D. citri* nymphs carrying parasitoid eggs or larvae (Fig. 3.3A).

This showed a direct relationship with infection as the smallest proportion of infection took place in nymphs carrying pupae of the parasitoid. These results clearly demonstrate that the advantage of prior residency was highly effective for the parasitoid. Interestingly, although the overall longevity of adult parasitoids emerging from healthy and fungus-infected *D. citri* nymphs were similar, there was a significant effect of isolate and developmental stage of the parasitoid. Longevity of adult parasitoids that emerged from *D. citri* nymphs carrying larvae of the parasitoid at the time of fungal inoculation was reduced compare with the longevity of adult parasitoids emerging from *D. citri* nymphs carrying the other parasitoid developmental stages at the time of fungal inoculation; this effect was more significant when *D. citri* nymphs were inoculated with the *M. anisopliae* isolate compared with the *B. bassiana* isolate (Fig. 3.4B). It is unclear why this specific developmental stage (larvae) was affected more than eggs and pupae.

Llandres et al., (2015) studying larvae of the parasitoid *Venturia canescens* Gravenhorst (Hymenoptera: Ichneumonidae), reported that later larval instars accumulated approximately 60 times more energy than early instar larvae, and 90% of this energy was later used during pupation. We hypothesise that when *D. citri* nymphs carrying larvae of the parasitoid were inoculated with fungi, that this energy accumulation process in the parasitoid larvae was severely negatively affected, perhaps due to the need to allocate more energy to overcoming infection than laying down reserves for pupation; this may have negatively affected overall development and lead to adults with reduced longevity compared to adults emerged from treatments where the parasitoids were inoculated with fungi as eggs or pupae.

More experiments are required to confirm our hypothesis. Similar results have been reported by other researchers where, in addition to reduced longevity, reductions in fertility and reproduction have also been observed as a result of sublethal fungal infections (**Tefera and Pringle 2003; Kaur et al., 2011**).

Overall, prior residency produced different outcomes for the two natural enemies evaluated here. Fungal infection was not significantly affected if infection was allowed to progress for at least 72 hours prior to parasitism; as a consequence the level of parasitism was reduced. However, parasitism showed a direct relationship between developmental stage of the parasitoid and infection; the older the parasitoid, the fewer *D. citri* nymphs became infected. Furthermore, the proportion of infected nymphs was reduced due to parasitoid host-feeding.

From a practical point of view, we suggest the further evaluation of isolates B1 and Ma129 in the field. To reduce the probability of a negative interaction between these isolates and *T. radiata*, it would be advisable to first release the parasitoids, exploiting the combined action of parasitism and host feeding.

Then, when a significant number of *D. citri* nymphs were carrying pupae, the fungi could be applied to infect remaining nymphs that were not parasitized, without affecting the emergence of *T. radiata*.

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Capítulo 4. Susceptibility of nymphs and adults of *Diaphorina citri* to the entomophathogenic fungus *Hirsutella citriformis*

Abstract

The susceptibility of nymphs and adults of *Diaphorina citri* to infection by *Hirsutella citriformis* was evaluated. We also studied the ability of adult *D. citri* that had been contaminated with fungal conidia, to transmit infection to nymphs. *Diaphorina citri* nymphs were more susceptible than adults. Adults were not able to transmit conidia to nymphs and cause infection. The implications of our results for the biological control of *D. citri* using *H. citriformis* are discussed.

Keywords: horizontal transmission, biological control, microbial control.

4.1 Introduction

Diaphorina citri Kuwayama (Hemiptera: Liviidae) is one of the most important pests in citrus orchards and is associated with transmission of Huanglongbing (HLB), a globally important citrus disease caused by the bacterium *Candidatus* Liberibacter asiaticus (**Bové, 2006**). The potential of entomopathogenic fungi for the biological control of *D. citri* has been studied under laboratory conditions (**e.g. Meyer et al., 2007; Avery et al., 2011; Orduño-Cruz et al., 2015a**).

Field observations made in citrus orchards in Mexico, showed that adult *D. citri* were naturally infected by the entomopathogenic fungus *Hirsutella citriformis* Speare but the infected nymphs were never observed (**Casique-Valdes et al., 2011; Orduño-Cruz et al., 2015b**); furthermore adult *D. citri* infected by *H. citriformis*, but not nymphs, have

also been observed under field conditions in other countries (**Subandiyah et al., 2000; Hall et al., 2012**) suggesting that this fungal species has potential as an effective biological control agent of *D. citri*. Despite this, very few studies have reported on the susceptibility of *D. citri* to *H. citriformis* under either laboratory or field conditions (**Meyer et al., 2007; Casique-Valdes et al., 2011; Orduño-Cruz et al., 2015a**).

A potential reason for the lack of extensive research on *H. citriformis* could be its slow *in vitro* development. For example, it has been reported that *H. citriformis* requires up to 40 days at 25 °C to produce aerial conidia, compared to other species of entomopathogenic fungi that require between 10 and 15 days (**Orduño-Cruz et al., 2015a, b**). In addition, **Orduño-Cruz et al. (2015b)** reported that when *H. citriformis* conidia are suspended in water, germination is reduced compared with dry conidia, leading to a reduced infection capacity.

It seems that inoculating *D. citri* with dry conidia of *H. citriformis* would be the most effective method to achieve infection. **Orduño-Cruz et al., (2015a**) achieved 100 % mortality of *D. citri* adults within six days when they were inoculated with dry conidia. Here we evaluated the relative susceptibility of nymphs and adults of *D. citri* to dry conidia of an isolate of *H. citriformis*. Secondly, we evaluated the capacity of *D. citri* adults to transmit dry conidia of *H. citriformis* to nymphs and initiate infection.

4.2 Material and methods

4.2.1 Fungal isolates and insect colonies

A monosporic isolate of *H. citriformis* (H1), previously studied by **Orduño-Cruz et al. (2015 b)** was used. Four 5 mm diameter fungus disks were cut from the growing edge of 45-day culture fungus growing on potato dextrose agar (PDA, BIOXON®, Becton

Dickinson de Mexico S.A. de C.V. Cuautitlan Izcalli, Mexico), and deposited into a 250 mL Erlenmeyer flask containing 50 mL of potato dextrose broth (PDB, BIOXON®, Becton Dickinson de Mexico S.A. de C.V. Cuautitlan Izcalli, Mexico) supplemented with yeast extract (1%). The flask was incubated at 120 r.p.m. for 12 days at 25 °C in a 12:12 light:dark regime. The concentration of blastospores in suspension were estimated using a haemocytometer and adjusted to 1 x 10^6 blastospores mL^{-1} using sterile distilled water. Ten ml of this suspension was inoculated into a plastic bag containing 125 g of sterile wheat bran. The wheat bran was sterilized twice at 120 °C for 15 min with an interval of 24 hours between each sterilization. Inoculated wheat bran bags were incubated at 25 °C in a 12:12 light regime for 30 days. Using sterile wheat bran as a substrate for growth of *H. citriformis* did not reduce the incubation time required (c.f. the studies of **Meyer et al., [2007]** using sterile rice), but it allowed us to easily produce sufficient material for experiments.

A different production lot was used for each occasion the experiment was run. On each occasion, three bags were produced, but usually one bag was more than sufficient.

Adults and nymphs of *D. citri* were provided by the Centro Nacional de Referencia de Control Biológico located in Colima, Mexico where a permanent colony is maintained on *Murraya paniculata* (L.) Jack (Sapindales: Rutaceae) plants under greenhouse conditions.

4.2.2 Relative susceptibility of *D. citri* nymphs and adults

Fourth-instar nymphs and adult *D citri* were collected using a fine camel hair brush and placed, in groups of 15, on to *M. paniculata* leaflets in 50 mm diameter Petri dishes. Each dish had a 30 mm diameter orifice in the base covered with mesh to allow

ventilation, and a filter paper, moistened with 0.5 mL of distilled water, in the lid; Petri dishes were incubated upside down. The petiole of the leaflet was immersed in a 2.0 mL Eppendorf type tube containing sterile distilled water to maintain turgor pressure (Fig. 4.1). On to each filter paper 0.5 g of wheat bran colonized with *H. citroformis* were placed and evenly distributed. The insects were maintained inside the Petri dish for 48 hours. After that, all insects were transferred to clean Petri dishes and further incubated for ten days.

Figure 4.1: Arena used for inoculation if *D. citri* adults and nymphs (Image Valdez-Carrazco, 2016).

Mortality was recorded every 24 hours and dead insects were placed in a 50 mm diameter Petri dish with damp filter paper and incubated at the same conditions for

further five days to encourage sporulation and synemmata production to confirm (or otherwise) fungal infection. The experiment was done using a completely randomized design with four pseudo-replicates of each treatment, and the complete experiment was repeated on three different occasions. On each occasion, a control treatment was set up as described above but without fungus.

4.2.3 Transmission of *H. citriformis* from adults to nymphs of *D. citri*

Using the same methodology as described previously, we first contaminated different groups of 15 *D. citri* adults for 48 hours; then, contaminated adults were introduced into new arenas as described above containing 15 nymphs of *D. citri* and incubated for a further 48 hours. After that time the adults were removed and transferred to clean Petri dishes, incubated for a further seven days under the the same conditions. Nymphs were not transferred but were incubated for seven days in the same arenas. In both cases, mortality was assessed every 24 hours. Fungal infection in dead insects was confirmed as described above.

The experiment was done using a completely randomized design with four pseudo-replicates of each treatment, and the complete experiment was repeated on three different occasions.

4.2.4 Statistical analyses

Mortality data for analyses only included those insects that died due to confirmed fungal infections. All experiments were analysed using logistic regression, assuming a binomial distribution and the number of dead insects as a proportion of the total number of insects tested. Prior to comparisons of treatment effects, data from the different replicates (occasions) were compared which allowed pooling of the data for treatment

comparisons. In the first experiment data from the control treatment were not included in the analysis as no mortality was recorded in adults and only 6% mortality was recorded for the nymphs, of which none were infected (i.e. they did not sporulate); then, we compared infection rates between nymphs and adults.

For the transmission experiment, data were analysed as described above. Data from control treatment were not included as, for both nymphs and adults, only 4% mortality was observed, of which none were infected (i.e. they did not sporulate). All analyses were done using GenStat v 8.0 (**Payne et al., 2005**).

4.3 Results and discussion

No differences were found amongst the three replicate assays to compare relative susceptibility of adults and nymphs of *D. citri* ($F_{2, 20}$ = 0.12, P=0.886), which allowed these data to be pooling for treatment comparisons; nymphs of *D. citri* were more susceptible to *H. citriformis* infection than adults ($F_{1, 20}$=14.86, P<0.001) (Fig. 4.2A). Previously, **Orduño-Cruz et al. (2105a)** found that *D. citri* adults were highly susceptible to *H. citriformis* (100% mortality), although they did not evaluate nymphs. We believe their results differed slightly from ours because they evaluated a different isolate (H2), but also, most importantly, they allowed the adults to be in contact with the fungus for six days and in our experiment the contact time was only 48 hours. It is not clear why, when *D. citri* nymphs are more susceptible to infection by *H. citriformis* than adults, that only adults are found infected in the field (**Subandiyah et al., 2000; Meyer et al., 2007; Hall et al., 2012**).

It is important to consider that, in our bioassay, we evaluated the physiological susceptibility of the nymphs when all conditions were favourable for infection, compared

with the ecological susceptibility that is observed under field conditions (**Roy and Pell, 2000**), where infection could be influenced by a range of biotic and abiotic factors.

We demonstrated that *D. citri* nymphs are susceptible to infection by *H. citriformis*, but the lack of fungal infection of nymphs in the field suggests that, in the field, there is a lack of contact between nymphs and infective *H. citriformis* conidia. However, the particular reasons for this remain to be investigated.

In the experiment to evaluate potential transmission from conidia-contaminated adult *D. citri* to nymphs no differences were found amongst replicates ($F_{2, 20}$ = 0.20, P=0.820) allowing the data to be pooled for treatment comparisons. The proportion of adult *D. citri* that became infected by *H. citriformis* was significantly greater than the proportion of nymphs that became infected ($F_{1, 20}$ = 93.96, P<0.001) (Fig. 4.2B). These results suggest that nymphs are unlikely to become infected after being in contact with contaminated adults. It is clear that the adults were heavily contaminated as we obtained a similar infection rate in adults as in the previous experiment (Fig.4.2B), but they failed to transmit the conidia to nymphs in sufficient numbers to cause infection. It has been reported that a 100% germination can be achieved in *H. citrioformis* dry conidia (not suspended in any carrier) after 48 h (**Orduño-Cruz et al., 2015b**); it is therefore likely that after 48 hours, when the adults were placed in contact with the nymphs, the majority of conidia attached to the adult's body had germinated and were in the process of penetration.

Therefore, only very few un-germinated conidia would be available to attach to and infect the nymphs. Further experiments using shorter inoculation times for the adults need to be evaluated to test this hypothesis.

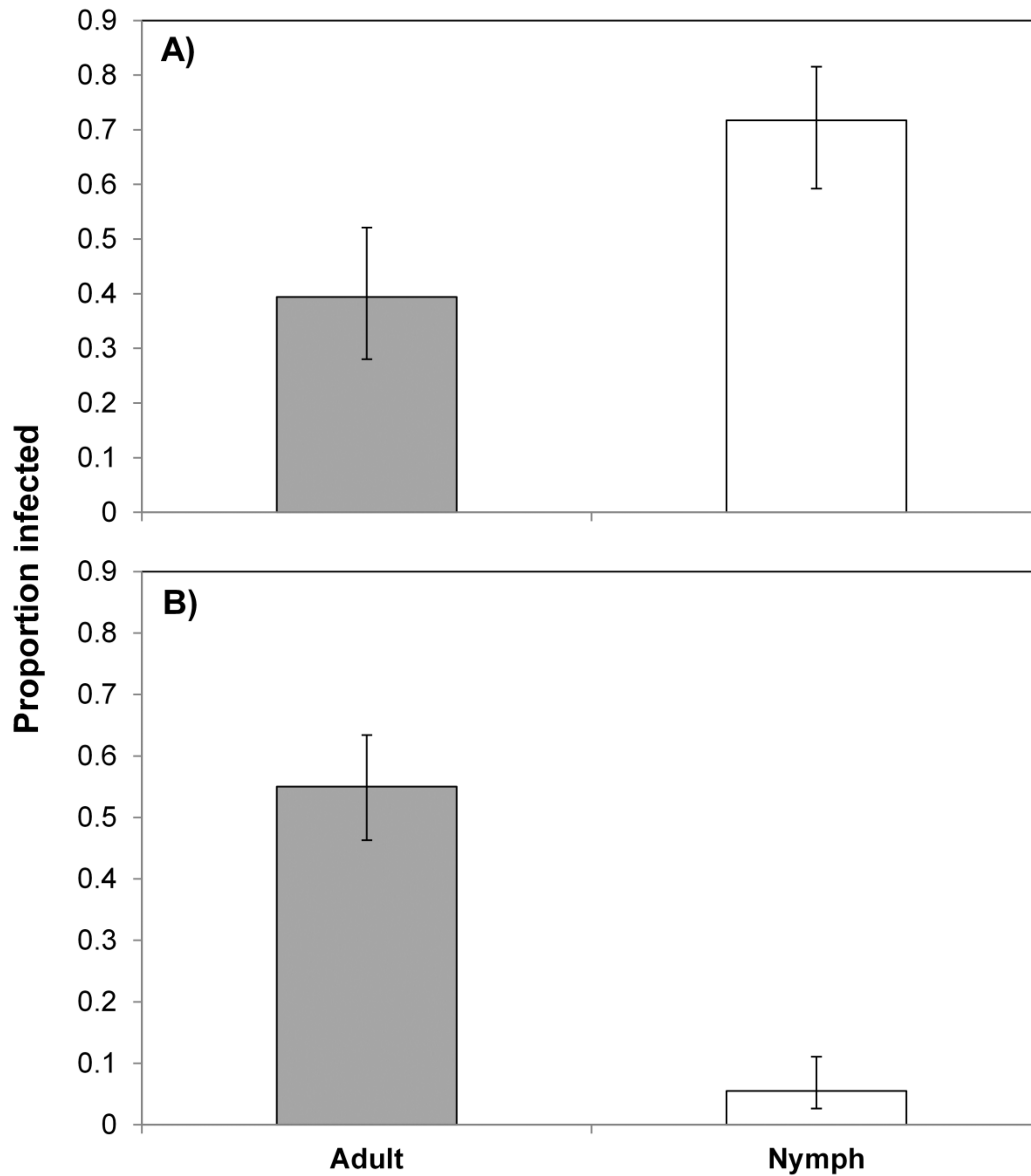

Figure 4.2: Proportion of *D. citri* adults and nymphs that became infected following direct inoculation with dried conidia (**A**), and when nymphs were placed in contact with conidia-contaminated adult *D. citri* (**B**). Error bars represents 95% confidence intervals back-transformed from the logistic scale.

Given these results it seems unlikely that transmission from adults to nymphs is possible; nevertheless, entomopathogenic fungi could still be useful if they were used in combination with parasitoids.

The *H. citroformis* would be useful for control of adult *D. citri* while the nymphs could be controlled by parasitoids such as *Tamarixia radiata* which has been found parasitizing *D. citri* nymphs in the field (**Cortez-Mondaca et al., 2009**). As the two natural enemies favour different life stages of *D. citri* intraguild predation could be avoided, although this needs further investigation.

In conclusion, we have shown that adults and nymphs of *D. citri* are both susceptible to infection by *H. citriformis* in the laboratory, and that the nymphs are actually the most susceptible. However, little infection could be achieved when nymphs were put in contact with fungal-contaminated adults and infections in nymphs are not observed in the field. The slow growth rate *in vitro* of *H. citriformis* may limit its use as a biological control agent of *D. citri*.

However, given that adult *D. citri*, which are not controlled by parasitoids, are highly susceptible to *H. citriformis* under field conditions, makes the potential for combined use of parasitoids and entomopathogenic fungi a strategy worth further consideration; by targeting different developments stages of *D. citri* intraguild predation could be avoided and overall pest control could be improved.

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Capítulo 5. Discusión General

Los hongos entomopatógenos y los parasitoides son una alternativa importante al control químico de plagas por sus bajos efectos a la salud humana y el ambiente. En las últimas dos décadas, agentes de control biológico y microbiano se incluyen dentro de la propuesta de Manejo Integrado de plagas exóticas en México como ha sido el caso de *M. hirsutus* y *D. citri* (**Valencia-Luna et al., 2007; Vizcarra-Valdez et al., 2007**). En este trabajo de tesis, se estudió la susceptibilidad de *D. citri* y su agente de control *T. radiata* a los hongos entomopatógenos y lo que ocurre cuando ambos enemigos naturales interaccionan.

Por lo anterior, en el segundo capítulo de está investigación se evaluó la virulencia de seis aislamientos de las especies de *B. bassiana* s.l., *M. anisopliae* s.s *e I. fumosorosea* en ninfas y adultos de *D. citri*, así como en adultos del parasitoide *T. radiata.* Los resultados muestran diferencias en la susceptibilidad entre adultos y ninfas de *D. citri* hacia los diferentes aislamientos evaulados. Las ninfas fueron más susceptibles a los aislamientos de hongos que los adultos. Los aislamientos MA129 y B1 fueron los más virulentos para ninfas y adultos, respectivamente. En diferentes trabajos se reporta la susceptibilidad de las ninfas de *D. citri* a la infección fúngica (**Ferreira-Pinto et al., 2012; Gandarilla-Pacheco et al., 2013**). Los resultados obtenidos confirman el potencial de los aislamientos MA129 y B1 para utilizarse dentro del manejo integrado de *D. citri* en ninfas y adultos.

La aplicación de los hongos entomopatógenos en nuestro país se dirige hacia los huertos comerciales. Donde se realizan actividades como monitoreo del adulto de *D. citri* con trampas amarillas, control químico y control biológico.

Sería importante considerar realizar monitoreos para estimar la población de las ninfas, el contar con esta información nos permitirá tomar decisiones cuando llevar a cabo la aplicación de hongos entomopatógenos para obtener mejores porcentajes de control. Diversos estudios indican que el control químico es la alternativa más utilizada para disminuir poblaciones del insecto vector *D. citri* (**Flores Villegas et al., 2016; Hall et al., 2013**), sin embargo esto conlleva problemas en la salud pública, resistencia de las poblaciones y disminución de los enemigos naturales ocasionando que los insecticidas sean ineficaces para prevenir la introducción y dispersión del HLB (**Hall et al., 2013**). Por está razón los hongos entomopatógenos son una alternativa efectiva pues ocasionan mortalidad en *D. citri* y tienen efectos subletales en la población como la reducción en oviposición, en la reproducción, disminuye su alimentación, la longevidad y produce malformaciones (**Kaur et al., 2011; Darbro et al., 2012; Pereira et al., 2011**). Dentro de este trabajo se observó que en las ninfas inoculadas con los aislamientos de *M. anisopliae,* estas presentaban problemas para desprenderse de la muda y les ocasionaba la muerte, también se presentarón malformaciones en las alas. Es necesario realizar investigaciones sobre los efectos subletales de los hongos entomopatógenos en las ninfas y adultos de *D. citri.*

Aunque el adulto del parasitoide *T. radiata* fue susceptible a la infección de los aislamientos utilizados, la virulencia fue menor en comparación con *D. citri.* Otros trabajos mencionan que los insectos benéficos son menos susceptibles a la infección

de los hongos entomopatógenos (**Furlong and Pell, 1996; Writght and Kennedy, 1996¸ Alma et al., 2007**).

En el manejo integrado del vector y la enfermedad del Huanglongbing en México las liberaciones de *T. radiata* se realizan en huertos abandonados de cítricos y zonas urbanas, pero es posible que este parasitoide se disperse a los huertos comerciales y podrían estar presentes en el momento de una aplicación con hongos entomopatógenos. Los resultados obtenidos sugieren que los hongos tienen potencial para ocasionar más mortalidad en la plaga que en *T. radiata*.

Por otro lado, cuando se liberan más de dos agentes de control biológico para el control de una plaga, es necesario contar con información de qué está ocurriendo con estas interacciones cuando compiten por un huésped. Los escenarios que se pueden presentar son: que el parasitoide disminuya su actividad de depredación y parasitación o por el contrario, que no repercuta en sus actividades como agente de control biológico. En el caso de *D. citri* y sus enemigos naturales (hongos y el parasitoide) se investigó como afecta la aplicación con los aislamientos MA129 y B1, con diferentes días de infección en la parasitación y depredación de *T. radiata* sobre ninfas de *D. citri*. Así mismo, se evaluó la emergencia del parasitoide *T. radiata* en diferentes etapas de desarrollo del parasitoide cuando son expuestos a los aislamientos anteriormente citados.

Cuando las ninfas de *D. citri* fueron inoculadas con los hongos y 0, 24 y 72 horas después éstas se expusieron a parasitismo por *T. radiata,* la proporción de ninfas parasitadas fue más baja cuando transcurrieron 72 h de infección de los hongos; mientras que la proporción de ninfas parasitadas más alta fue cuando pasaron 24 h de

infección de los hongos. **Tamayo-Mejia et al., (2015)** señalan que al transcurrir los días de la infección de *B. bassiana* en ninfas de *B. cockerelli*, la proporción de ninfas parasitadas con *T. triozae* disminuye e inclusive no se observa parasitismo a los 6 días de infección. Respecto a las ninfas depredadas por *T. radiata* se encontró que no existieron diferencias al transcurrir la infección. El parasitoide *T. radiata* es una especie sinovigénica y es común que las hembras consuman proteína para madurar sus huevos antes de ovipositar (**Bernal, 2007; Hoy et al., 2006**), lo anterior lo obliga alimentarse de ninfas sanas o enfermas. El ser un parasitoide con capacidad de alimentarse de su huésped aumenta la tasa de mortalidad en las poblaciones de *D. citri*. Por otra parte, cuando los diferentes estados de desarrollo de *T. radiata* como huevo, larva y pupa se expusieron a los hongos entomopatógenos, se encontró que todos los estados de desarrollo del parasitoide son susceptibles a la infección de hongos entomopatógenos. No obstante, cuando *T. radiata* se encontraba en fase de pupa, se obtuvo la menor proporción de infección comparada con el huevo, el cual presentó la mayor proporción de infección. Respecto a la emergencia de adultos de *T. radiata,* está fue mayor a partir de pupas inoculadas con hongos entomopatógenos.

En otros trabajos se ha reportado que la emergencia de parasitoides es afectada por los hongos entomopatógenos en condiciones de laboratorio (**Castillo et al., 2009; Martins et al., 2014; Tamayo-Mejía et al., 2016**), pero cuando el parasitoide tiene más avance en su desarrollo, la infección por hongos entomopatógenos tiene un bajo efecto en la emergencia del adulto parasitoide (**Tamayo-Mejía et al., 2016**). Lo anterior sugiere que la susceptibilidad a la infección por hongos entomopatógenos puede depender en gran medida del desarrollo de insectos (**Liu et al., 2014**). La

supervivencia de los adultos de *T. radiata* en los tratamientos de ninfas de *D. citri* inoculadas sólo con hongos fue de 18.97 (± 0.906) días; mientras que para ninfas de *D. citri* que sólo fueron expuestas a *T. radiata* la superviviencia del parasitoide emergido fue mayor, con 23.09 (± 0.530) días. Por otra parte, es necesario tener conocimiento de los efectos subletales de los hongos entomopatógenos en los parasitoides emergidos de ninfas infectadas, como por ejemplo en su fertilidad, proporción de sexos e inclusive alteraciones morfológicas. Una mayor información acerca de las interacciones entre insectos plagas y sus agentes de control biológico, permitirá visualizar si son compatibles, realizar un control independiente y/o manejar tiempos para evitar interacciones antagónicas.

Un hongo entomopatógeno que ha sido encontrado infectando grandes proporciones de adultos de *D. citri* en campo es *H. citriformis* (**Casiques-Valdes et al., 2011; KHIC observación personal**). Sin embargo, ha sido poco estudiado, probablemente por las diferencias en velocidad de crecimiento y producción de conidios comparada con otros hongos entomopatógenos (**Orduño-Cruz et al., 2015 a**).

Por lo que en el cuarto capítulo se evaluó la susceptibilidad de ninfas y adultos de *D. citri* a un aislamiento de *H. citriformis* en condiciones de laboratorio, así como la transmisión de *H. citriformis* de adultos inoculados hacia ninfas de *D. citri*. Otros autores ya han realizado investigación acerca de la virulencia de *H. citriformis* en adultos de *D. citri* (**Casique-Valdes et al., 2011; Orduño-Cruz et al., 2015 b**).

En los resultados de esta investigación se encontró que tanto ninfas como adultos de *D. citri* son susceptibles a la infección de *H. citriformis*. El estado ninfal fue el más

susceptible a *H. citriformis*. **Goettel (1994)** señala que ciertas especies de insectos pueden ser infectados fácilmente con hongos entomopatógenos en condiciones de laboratorio. **Pérez-González et al., (2015)** reporta en su trabajo que las ninfas en comparación con los adultos fueron menos susceptibles a la infección de *H. citriformis*, lo anterior difiere de nuestros resultados y posiblemente se deba a que se utilizaron aislamientos diferentes y la inoculación fue con esporas en suspensión. Por otro lado, la transmisión de *H. citriformis* a partir de adultos de *D. citri* contaminados hacia sus ninfas fue muy baja. Estos resultados indican que hay poca probabilidad de que las ninfas logren infectarse después de estar en contacto con adultos contaminados. Posiblemente el tiempo que se expuso a las ninfas con los adultos contaminados fue insuficiente para que se diera la infección. De acuerdo con **Orduño-Cruz et al., (2015 a)**, el conidio seco germina a las 48 horas y posiblemente el tiempo que estuvieron en contacto las ninfas con los adultos contaminados, aún no germinaba el conidio. Por tanto, se recomienda realizar futuras investigaciones donde se exponga al adulto de *D. citri* a *H. citriformis* en diferentes tiempos.

En conclusión, es posible la combinación de los aislamientos B1 y MA129 y el parasitoide *T. radiata* para el control biológico de *D. citri* en poblaciones de campo, aunque bajo ciertas consideraciones, como el hecho de que al tratarse de una especie plaga que actua como vector y donde los umbrales de acción son muy bajos, por lo que es importante tomar en cuenta que para el manejo de esta especie plaga, la recomedación de usar en forma combinada estos agentes de control biológico se debe hacer como un componente dentro de un programa de manejo intergado de plagas,

con el objeto de mantener las poblaciones aún más bajas de *D. citri*, en regiones citrícolas donde por ejemplo se está aplicando el programa ARCO.

Por otro lado, para minimizar una interacción negativa para cualquiera de los dos agentes de control biológico, sería recomendable liberar primero los parasitoides para que impacte en las poblaciones de *D. citri* por la acción combinada del parasitismo y la alimentación, y posteriormente, cuando un número significativo de *D. citri* contenga pupas del parasitoide, los hongos se podrían aplicar para infectar ninfas restantes que no fueron parasitados, sin afectar la emergencia de *T. radiata*. Otra opción sería que primero se aplicarán los entomopatógenos que tuvieron poco efecto sobre *T. radiata* y después liberar *T. radiata*, ya que el parasitoide puede discriminar al parasitar, entre una ninfa sana o infectada, o como adulto puede alimentarse, de cualquier ninfa de *D. citri*, sana o infectada.

Para *H. citriformis* se debe realizar más estudios de producción masiva y en un futuro buscar alternativas para la autoinoculación de esporas en seco dentro de los agroecosistemas.

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