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VARIACIÓN GENÉTICA EN POBLACIONES DE *Tetranychus urticae* (ACARI: TETRANYCHIDAE) Y SUSCEPTIBILIDAD A HONGOS ENTOMOPATÓGENOS

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VARIACIÓN GENÉTICA EN POBLACIONES DE *Tetranychus urticae* (ACARI: TETRANYCHIDAE) Y SUSCEPTIBILIDAD A HONGOS ENTOMOPATÓGENOS

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RESUMEN

Se estudió la relación potencial entre la estructura genética poblacional de *Tetranychus urticae* Koch y la susceptibilidad a la infección por hongos. Se tomaron muestras de adultos de *T. urticae* de 10 lugares de muestreo diferentes, seis para *Rubus ulmifolius* Schoot (zarzamora) y cuatro para *R. idaeus* L. (frambuesa). Usando las secuencias de un fragmento de la subunidad mitocondrial de la citocromo oxidasa I (COI), se determinaron las relaciones filogenéticas y la estructura de la población genética usando los análisis de redes de haplotipos y un análisis de varianza molecular (AMOVA). Con base a estos resultados, se seleccionaron cuatro poblaciones para estudiar la susceptibilidad contra aislamientos de las especies *Beauveria bassiana* (Bals.-Criv.) Vuill., *Metarhizium anisopliae* (Metschn.) Sorokin, *Lecanicillium lecanii* (Zimm.) Zare y W. Gams e *Isaria fumosorosea* Wise. Se estudiaron dos poblaciones de frambuesa (var. '7-UDV' y var. '8-UDV') y dos de zarzamora (var. 'Tupy' y var. '2-UDV'). El análisis de red de haplotipos mostró la existencia de ocho haplotipos, la mayor diversidad genética se encontró para la población de frambuesa '7-UDV' con cinco haplotipos y las poblaciones restantes con uno o dos haplotipos. El análisis AMOVA mostró que ni la planta hospedera ni el origen geográfico explicaron la variación genética total. La población más susceptible a la infección por hongos fue '2-UDV' seguido de 'Tupy', ambos de zarzamora. Los aislamientos que causaron más mortalidad fueron *M. anisopliae* y *B. bassiana*. Las colonias menos susceptibles fueron '7-UDV' y '8-UDV', ambas de frambuesa, y aunque todos los aislamientos causaron infección, no se obtuvieron diferencias entre los aislamientos. Los resultados mostraron que la planta huésped tuvo un papel importante en la susceptibilidad a la infección por hongos, en comparación con la diversidad genética. Se discute el efecto de los metabolitos producidos por estas plantas hospedantes en los resultados.

Palabras clave: ácaro de dos puntos, hongos entomopatógenos, zarzamora, frambuesa.

GENETIC VARIATION IN *Tetranychus urticae* (ACARI: TETRANYCHIDAE) POPULATIONS AND SUSCEPTIBILITY TO ENTOMOPATHOGENIC FUNGI

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Colegio de Postgraduados, 2018

ABSTRACT

We studied potential relationships between the genetic structure of populations of *Tetranychus urticae* Koch and their susceptibility to fungal infection. We sampled adult *T. urticae* from ten locations, comprising six *Rubus ulmifolius* Schoot (blackberry) orchards and four *R. idaeus* L. (raspberry) orchards. Using sequence information from a fragment of the mitochondrial cytochrome oxidase subunit I (COI), phylogenetic relationships and genetic population structure of each group were determined using haplotype network analyses and an analysis of molecular variance (AMOVA). Based on these results, four populations were selected to compare their susceptibility to isolates of *Beauveria bassiana* (Bals.-Criv.) Vuill., *Metarhizium anisopliae* (Metschn.) Sorokīn, *Lecanicillium lecanii* (Zimm.) Zare & W. Gams and *Isaria fumosorosea* Wise. Two populations from raspberry (var. '7-UDV' and var. '8-UDV') and two from blackberry (var. 'Tupy' and var. '2-UDV') were studied. Haplotype network analysis identified eight haplotypes. The greatest genetic diversity was found in the '7-UDV' raspberry population which had five haplotypes; the remaining populations had one or two haplotypes each. The AMOVA analysis showed that neither host plant nor geographical origin explained all the genetic variation. The '2-UDV' population was the most susceptible to fungal infection, followed by 'Tupy', both of which were from blackberry. The fungal isolates that caused greater mortality in *T. urticae* were *M. anisopliae* and *B. bassiana*. In the least susceptible populations ('7-UDV' and '8-UDV', both from raspberry), all isolates still caused some mortality, but there were no differences amongst the isolates. Our results suggest that host plant of origin plays a more important role than genetic diversity in the susceptibility of *T. urticae* to fungal infection. The effect of metabolites produced by these host plants is discussed in relation to this.

Keywords: two spotted spider mite, entomopathogenic fungi, raspberry, blackberry.

A mis papás, Irma y Miguel

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INTRODUCCIÓN GENERAL

El ácaro de dos puntos (TSSM) *Tetranychus urticae* Koch (Acari: Tetranychidae) es una plaga cosmopolita que se alimenta de más de 1100 especies de plantas pertenecientes a más de 140 familias de plantas (Grbic *et al*., 2011; Migeon y Dorkeld, 2017) incluyendo árboles frutales, frutillas, vegetales, granos y plantas ornamentales (Attia *et al*., 2013; Van Leeuwen *et al*., 2015). TSSM causa manchas amarillentas y necróticas en las hojas y en caso de infestación severa puede causar defoliación que conduce a pérdidas significativas de rendimiento.

En México destacan los cultivos de zarzamora (*Rubus ulmifolius* Schott) y frambuesa (*Rubus idaeus* L.) debido a su alta demanda en mercados internacionales, creación de empleo y valor económico de más de 650 millones de dólares estadounidenses en conjunto (SIAP, 2016). El estado de Michoacán es el principal productor con 70.40% del área plantada, seguido de Jalisco y Baja California con 25.28% y 3.20% respectivamente (SIAP, 2016). Sin embargo TSSM representa uno de los principales problemas fitosanitarios en la producción de estos cultivos.

La aplicación de acaricidas es el método de control más común de TSSM en la producción de frutillas en México; no obstante, debido al corto ciclo de vida, abundante progenie y capacidad de reproducción partenogenética de este ácaro, la aplicación de estos compuestos puede ser muy frecuente; como consecuencia, se elevan los costos de producción, se contamina el ambiente, el cual tiene efectos negativos a la salud humana y se desarrolla rápidamente resistencia a los plaguicidas (Van Leeuwen *et al*., 2010 y 2015). A la fecha está documentado que TSSM es resistente a 93 ingredientes activos, por lo que este ácaro plaga requiere de alternativas al control químico para su control (Van Leeuwen *et al*., 2015).

Dentro de las principales alternativas, se encuentra el control biológico con ácaros depredadores de la familia Phytoseiidae (Van Lenteren, 2012; Ghazy *et al*., 2016); aplicaciones de extractos de plantas y aceites esenciales (Attia *et al*., 2013) y aplicaciones de hongos entomopatógenos, donde destacan comercialmente *Beauveria bassiana* (Bals.-Criv.) Vuill., *Metarhizium anisopliae* (Metschn.) Sorokīn e *Isaria fumosorosea* Wise. Estos últimos han tenido gran éxito debido a los bajos costos de producción, alta eficiencia, y por ser amigables con el ambiente (Maniania *et al*., 2008; Khan *et al*. 2012; Gul *et al*., 2014).

Las regiones productoras de zarzamora y frambuesa en México se ubican en diferentes agroecosistemas; por lo que las variedades que se producen y el control de plagas que se aplican varían según los productores: Manejo Integrado de Plagas (MIP) (basado principalmente en control químico aunque también se aplican métodos menos agresivos) o Control Biológico. Estas diferencias en los métodos de control en combinación con el efecto de las plantas hospederas podrían tener un efecto en la estructura genética poblacional de TSSM.

Diversos estudios en ácaros reportan el efecto de distintos factores en la estructura poblacional, tales como asociaciones a las plantas hospederas (Navajas, 1998; Nishimura *et al*., 2005; Guzmán-Valencia *et al*., 2017), resistencia a plaguicidas (Uesugi *et al*., 2009; Hada *et al*., 2016), prácticas agrícolas (Pascual-Ruiz *et al*., 2014; Hada *et al*., 2016), separación geográfica (Xie *et al*., 2006; Carbonnelle *et al*., 2007; Guzmán-Valencia *et al*., 2014) y el efecto de los endosimbiontes (Zhang *et al*., 2013; Chen *et al*., 2016).

La aplicación de marcadores moleculares, precisando de una vez el estudio de ITS2, COI y microsatélites se ha vuelto indispensable en estudios filogenéticos, estructuras

genéticas de poblaciones e identificaciones taxonómicas de ácaros de la familia Tetranychidae (Navajas *et al*., 1998; Navajas y Fenton, 2000; Hinomoto *et al*., 2001; Navajas *et al*., 2002; Cruickshank, 2002; Navajas y Boursot, 2003; Dabert, 2006; Carbonnelle *et al*., 2007; Ros y Breeuwer, 2007; de Mendonça *et al*., 2011; Sun *et al*., 2012; Pascual-Ruiz *et al*., 2014). La parte central de la región COI mitocondrial se ha utilizado con frecuencia para estudiar la variación intra e interespecífica de tetraniquidos (Navajas *et al*., 1998; Toda *et al*., 2000; Hinomoto *et al*., 2001; Hinomoto *et al*., 2007; Ros y Breeuwer, 2007; Gotoh *et al*., 2009).

Ros y Breeuwer (2007) realizaron un análisis filogenético de todas las secuencias de la región COI de TSSM existentes en las bases de datos y encontraron una variación importante entre los tetraniquidos estudiados, especialmente una variación intraespecífica, por lo que recomiendan el uso de esta región COI para este propósito. Por otro lado, Matsuda *et al*. (2012, 2013) señalan que casi todas las especies japonesas de *Olygonychus* y *Tetranychus* pueden identificarse a través de la región COI, por lo que consideran que esta región es útil para estudios de variación interespecífica. En resumen, estos autores encuentran la región COI como una importante herramienta para determinar la variación genética en la estructura poblaciones de *T. urticae*.

Trabajos previos han estudiado la diversidad genética y el método de control de *T. urticae*. Pascual-Ruiz *et al*. (2014) lo hicieron por medio de marcadores moleculares en cultivos de clementinas en España, bajo manejo integrado de plagas (MIP) y bajo manejo orgánico de plagas (MOP), en un lapso de cuatro años. Ellos encontraron que las dinámicas poblacionales y la variación genética se ven mayormente afectadas por el MIP comparado con el MOP. Por otro lado, Hada *et al*. (2016) investigaron la estructura genética de *T. urticae* en

cultivo de manzana y reportan que las aplicaciones frecuentes de acaricidas generan una disminución en la variación genética.

Sin embargo, para nuestro conocimiento, no hay estudios previos de la diversidad genética de *T. urticae* y la susceptibilidad a hongos entomopatógenos. El presente trabajo estudió la posible relación entre la genética de la estructura poblacional y la susceptibilidad a la infección por hongos entomopatógenos en poblaciones de *T. urticae* obtenidas de zarzamora y frambuesa colectadas en diferentes regiones productoras de México.

Objetivos e hipótesis

Objetivo general

Estudiar el efecto de la diversidad genética y la planta hospedera de poblaciones de *Tetranychus urticae* de zarzamora y frambuesa en la susceptibilidad a la infección de hongos entomopatógenos.

Objetivos específicos

- Determinar la variación genética de poblaciones de *T. urticae* en zonas productoras de zarzamora y frambuesa de Michoacán, Jalisco, Colima, y Baja California.
- Evaluar los niveles de infección que ocasionan aislamientos de los hongos entomopatógenos *Beauveria bassiana*, *Metarhizium anisopli*a, *Isaria fumosorosea y Lecanicilium lecanii* en poblaciones seleccionadas de *T. urticae.*

Hipótesis

Poblaciones de *T. urticae* con mayor diversidad genética serán menos susceptibles a la infección por hongos entomopatógenos.

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CHAPTER 1. INTERACTIONS BETWEEN HOST GENETIC DIVERSITY AND HOST PLANT INFLUENCE THE SUSCEPTIBILITY OF *TETRANYCHUS URTICAE* KOCH POPULATIONS TO FUNGAL INFECTION[1]

ABSTRACT

We studied potential relationships between the genetic structure of populations of *Tetranychus urticae* Koch and their susceptibility to fungal infection. We sampled adult *T. urticae* from ten locations, comprising six *Rubus ulmifolius* Schoot (blackberry) orchards and four *R. idaeus* L. (raspberry) orchards. Using sequence information from a fragment of the mitochondrial cytochrome oxidase subunit I (COI), phylogenetic relationships and genetic population structure of each group were determined using haplotype network analyses and an analysis of molecular variance (AMOVA). Based on these results, four populations were selected to compare their susceptibility to isolates of *Beauveria bassiana* (Bals.-Criv.) Vuill., *Metarhizium anisopliae* (Metschn.) Sorokīn, *Lecanicillium lecanii* (Zimm.) Zare & W. Gams and *Isaria fumosorosea* Wise. Two populations from raspberry (var. '7-UDV' and var. '8-UDV') and two from blackberry (var. 'Tupy' and var. '2-UDV') were studied. Haplotype network analysis identified eight haplotypes. The greatest genetic diversity was found in the '7-UDV' raspberry population which had five haplotypes; the remaining populations had one or two haplotypes each. The AMOVA analysis showed that neither host plant nor geographical origin explained all the genetic variation. The '2-UDV' population was the most susceptible to fungal infection, followed by 'Tupy', both of which were from blackberry. The

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fungal isolates that caused greater mortality in *T. urticae* were *M. anisopliae* and *B. bassiana*. In the least susceptible populations ('7-UDV' and '8-UDV', both from raspberry), all isolates still caused some mortality, but there were no differences amongst the isolates. Our results suggest that host plant of origin plays a more important role than genetic diversity in the susceptibility of *T. urticae* to fungal infection. The effect of metabolites produced by these host plants is discussed in relation to this.

Keywords: Two-spotted spider mite, entomopathogenic fungi, raspberry, blackberry

1.1 Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), is a cosmopolitan phytophagous pest that feeds on more than 1100 plant species from more than 140 plant families (Grbic et al., 2011; Migeon and Dorkeld, 2017). These include fruit trees, berries, vegetables, grains and ornamentals (Attia et al., 2013; Van Leeuwen et al., 2015). This mite causes yellowing and necrotic spots on leaves and, in cases of severe infestation, they cause defoliation leading to significant yield loss.

In Mexico, blackberry (*Rubus ulmifolius* Schott) and raspberry (*Rubus idaeus* L.) (Rosales: Rosaceae) crops are economically important; high demand from international markets and the associated jobs created represent an estimated economic value of more than 650 million US dollars (SIAP, 2016). The two-spotted spider mite is one of the most important factors affecting the yield of these crops. The state of Michoacan is the main producer with 70.4 % of the total area planted with blackberry and raspberry, followed by Jalisco and Baja California with 25.3 % and 3.2 %, respectively (SIAP, 2016).

In Mexican berry production, application of acaricides is the most widely used control method for this mite. However, due to the mite's short life cycle, abundant progeny and parthenogenetic reproduction, application of these compounds is often very frequent; as a result, production costs rise, the environment can be polluted, and pesticide resistance can develop rapidly (Van Leeuwen et al., 2010, 2015). For these reasons, alternative methods are increasingly being developed, all be it on a smaller scale. These include the use of predatory mites, plant extracts and entomopathogenic fungi (Maniania et al., 2008; Khan et al., 2012; Attia et al., 2013; Gul et al., 2014; Ghazy et al., 2016).

The blackberry and raspberry-producing regions in Mexico are located in different geographical zones with different climates and agri-environments. Furthermore, the varieties grown and the pest control strategies used varies according to producer. This combination of differences in control methods and host plant varieties may have an effect on the genetic population structure of *T. urticae*. Several factors are known to influence the genetic population structure of mites and include: host plant associations (Navajas, 1998; Nishimura et al., 2005; Guzmán-Valencia et al., 2017); pesticide resistance (Uesugi et al., 2009; Hada et al., 2016); agricultural practices (Pascual-Ruiz et al., 2014; Hada et al., 2016); geographical separation (Xie et al., 2006; Carbonnelle et al., 2007; Guzmán-Valencia et al., 2014); and the presence of endosymbionts (Zhang et al., 2013; Chen et al., 2016).

The use of microsatellites and molecular markers for regions within the nuclear internal transcribed spacer (ITS2) and the mitochondrial cytochrome oxidase subunit I (COI), has become indispensable in phylogenetic studies, population genetic studies and taxonomic identifications of mites species within the family Tetranychidae (Navajas et al.,1998; Navajas

and Fenton, 2000; Hinomoto et al., 2001; Navajas et al., 2002; Cruickshank, 2002; Navajas and Boursot, 2003; Dabert, 2006; Carbonnelle et al., 2007; Ros and Breeuwer, 2007; de Mendonça et al., 2011; Sun et al., 2012; Pascual-Ruiz et al., 2014). The COI gene has been used frequently to study intra and interspecific variation amongst tetranychids (Navajas et al., 1998; Toda et al., 2000; Hinomoto et al., 2001, 2007; Ros and Breeuwer, 2007; Gotoh et al., 2009). A number of studies have reported the convenience of using COI partial sequences to identify species within the genus *Tetranychus* (Hinomoto et al., 2001; Matsuda et al., 2012, 2013). COI sequences are also known to have great genetic diversity amongst *Tetranychus* species (Ros and Breeuwer, 2007). These attributes suggest that COI sequence information is a valuable tool for determining the genetic population structure of *T. urticae* populations.

Previous studies have identified relationships between genetic diversity in *T. urticae* populations and control methods used, specifically the use of different acaricides (Pascual-Ruiz et al., 2014; Hada et al., 2016). However, to the best of our knowledge, no previous studies have related genetic diversity in *T. urticae* populations with susceptibility to fungal infection. Therefore, we have studied the possible relationships between genetic population structure and susceptibility to fungal infection in *T. urticae* populations obtained from blackberry and raspberry crops from different geographical regions in Mexico.

1.2 Material and methods

1.2.1 Collection sites

Populations of *T. urticae* were collected from blackberry and raspberry orchards in Michoacan, Jalisco, Colima and Baja California between May and October 2017 (Table 1).

The methodology was the same in each orchard. Within each orchard (approx. 1 Ha), five sampling areas were selected at the four corners and the centre. Ten leaves (five from the upper part and five from the lower part of the plant) were taken from each area, representing a total of 50 leaves per orchard. Collected leaves were deposited in a cool box inside labelled plastic bags and transported to the laboratory. Using a stereomicroscope, each leaf was carefully observed and between 20 and 50 female adults mites collected and deposited into 1.5 mL Eppendorf type tubes and stored at -20 °C prior to molecular identification/ genetic population evaluation (never for more than one month). At the same time, approx. five adults (mixed sex) were deposited in 1.5 mL Eppendorf type tubes containing 70% ethanol for morphological identification.

1.2.2 Morphological identification, molecular identification, and genetic population evaluation of mites

For morphological identification, females and males were mounted in Hoyer's medium (Krantz and Walter, 2009), and identified using the taxonomic keys of Baker and Tuttle (1994) and Bolland et al., (1998).

For molecular identification, DNA from each mite was extracted using the DNeasy Blood & Tissue kit (QIAGEN, Germantown, MD, USA) following the manufacturer´s instructions. Extracted DNA was stored at -20 °C until it was used in PCR reactions. Partial sequences of the COI gene were obtained using the primers 5'-AAGAGGAGGAGGAGACCCAA-3 'and 5'-AAACCTCTAAAAATAGCGAATACAGC-3' (Hinomoto and Takafuji, 2001). The PCR reaction was done in a final volume of 30 µL containing 3 µL of 10x PCR buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 15mM $MgCl_2$, pH 8.7), 2mM

$MgCl_2$ (QIAGEN), 0.2mM dNTPs, 20 pmol of each primer, 0.5 U of Taq DNA polymerase (QIAGEN, GmbH, Hilden, Germany) and 5 µl of the mite DNA solution (approx. 40 ng of DNA). Amplifications were done in a MyCycler thermocycler (BIORAD Laboratories Inc., Hercules, CA, USA) using the following conditions: one cycle of 3 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C, 2 min at 72 °C, and a final extension of 10 min at 72 °C. The PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide (10 mg mL^{-1}) in 1X TAE and photographed. All PCR products were sent to the company Macrogen Inc. (South Korea) for direct sequencing.

1.2.3 Phylogeny reconstruction

All sequences were edited using the program BioEdit v 7.2.6.1 (Hall, 1999). Multiple alignments of the sequences were made using the ClustalW program (Thompson et al., 1994) implemented in BioEdit. After aligning and cutting sequences, the final length of the COI sequence for *T. urticae* was 383 bp (79 sequences). GenBank accession numbers for *T. urticae* are listed in Table 1.

Sequences were analysed using maximum likelihood in Molecular Evolutionary Genetic Analysis (MEGA) ver. 6.06 for Windows, with the Close-Neighbour-Interchange algorithm (Tamura et al., 2011). The robustness of branches was estimated by bootstrap analysis with 1000 repeated samples from the data (Felsenstein, 1985). In addition, sequences were retrieved from GenBank and used as references, specifically: *T. urticae* (green form [GF]; AB066453) and *T. urticae* (red form [RF]; AB066463) (Hinomoto et al., 2001); *Tetranychus kanzawai* Kishida (AB079036) (Hinomoto and Takafuji, 2001); *Tetranychus truncatus* Ehara (AB257315) (Hinomoto et al., 2007); *Tetranychus mcdanieli* McGregor

(X80857), *Tetranychus pacificus* McGregor (X80858) and *Tetranychus neocaledonicus* Andre (X80859) (Navajas et al., 1996). Sequences for *Panonychus citri* McGregor (AB041252) and *Panonychus mori* Yokoyama (AB041256) (Toda et al., 2000) were used as outgroups. Genetic differences amongst haplotypes were detected in an MP network (Templeton et al., 1992) using TCS v. 1.21 (Clement et al., 2000). The connection limit amongst haplotypes was set to the default value of 95%. The partition of genetic variation between groups (plant species), amongst populations within each group (varieties) and finally within populations, was assessed by analysis of molecular variance (AMOVA), estimated by computing F-statistics using Arlequin v. 3.5 (Excoffier and Lischer, 2010) with 10,000 permutations.

1.2.4 Susceptibility of T. urticae to fungal infection

1.2.4.1 Tetranychus urticae colonies

From the ten populations studied at the molecular level, four were selected based on the host plant and the genetic diversity results. Two populations from raspberry ('7-UDV' and '8-UDV') and two from blackberry ('2-UDV' and 'Tupy') were selected (Table 1). To establish the experimental colonies, mites from each selected site were collected again and transported to the laboratory. Using a stereomicroscope, female mites were collected and immediately placed on *Phaseolus vulgaris* bean plants (var. Flor de Mayo). Colonies were maintained in controlled environment (CE) rooms at 25 °C and 70% RH and a 12:12 light regime. Two wooden cages of 40×40×40 cm with insect proof mesh per mite colony were used and each colony was maintained in a separate CE room to avoid cross contamination. Colonies were maintained on bean plants, which were replaced as required. Clean plants were

grown in a separate CE room in 1 L containers in a mixture of sterile peat moss, vermiculite and soil in equal proportions as the substrate. Plants were watered using tap water every three days.

1.2.4.2 Fungal isolates

Four isolates, each from a different species were used (Table 2). Isolates were grown on plates of Sabouraud Dextrose Agar (SDA) at 25 °C for 14 days in total darkness. Conidia were harvested by scraping them from sporulating colonies using a sterile metal spatula and depositing them into a 50 mL centrifuge tube containing 30 mL of a 0.03% (v/v) Tween 80 solution. Tubes containing conidial suspensions were vortexed for ten minutes to separate the conidia from the mycelium and filtered through a double layer of sterile gauze into a new 50 mL centrifuge tube to remove hyphae. Concentrations of conidial suspensions were estimated using a haemocytometer (Hausser Scientific, USA), and adjusted to a final concentration of $1x10^8$ conidia mL^{-1}.

1.2.4.3 Experimental setup

The methodology for each fungal isolate and mite population was similar. Groups of 20 adult females were inoculated with 5 mL of a $1x10^8$ conidia mL^{-1} suspension. To achieve this the females were placed in a Petri dish containing a fresh bean leaf (experimental arena). Each arena were prepared by embedding a 40 mm diameter leaf disk into 8 mL water-agar (3%) contained in a 40 diameter Petri dish base. Leaf disks were placed abaxial side uppermost ensuring the entire border of each leaf disk was immersed in the water-agar. Inoculations with conidial suspensions were made using a spray tower fitted with a cone spray nozzle (Spraying Systems Co. Wheaton, IL, USA) attached to an air compressor at 10 p.s.i.

Each group of 20 female mites were inoculated with 5 mL of the conidial suspension; between isolates, the spray tower and the nozzle were cleaned first by spraying 5 mL of 96% ethanol followed by two sprays of 5 mL each of sterile distilled water. A control treatment was included where mites were sprayed only with 5 mL of 0.03% Tween 80 solution.

After inoculation, all arenas were incubated at 25 °C, 70% RH and a light regime of 12:12. Mites from all treatments were transferred to clean arenas after 24 hours incubation and again after 72 hours inoculation. These transfers ensured that good quality leaves were maintained for the mites, and allowed us to remove any eggs deposited before they could hatch. Mortality was recorded every 24 h for 5 d. Dead mites were transferred to 40 mm diameter Petri dishes containing sterile 2% water-agar, and incubated for a further 3 d under the same experimental conditions. This encouraged sporulation and allowed us to confirm whether mortality had been caused by fungal infection, or not. Prior to experimentation, viability tests were done on the conidia and on all occasions the germination rate was above 95%.

Due to space restrictions, only two mite colonies could be maintained simultaneously. Therefore, experiments were first done using colonies derived from ‘Tupy’ and ‘2-UDV’, followed by colonies derived from ‘7-UDV’ and ‘8-UDV’. Each experiment was done using a completely randomized design with two replicates per treatment (isolates and control), and the complete experiment was repeated on four different occasions.

1.2.4.4 Statistical analyses

We used residual maximum likelihood (REML) meta-analysis for statistical analysis. REML meta-analysis is used to combine different experiments with similar treatment

structure and with some treatments in common across the experiments (Payne et al., 2005). Briefly, the error model was first estimated for each experiment using Liner Mixed Models. Then, using REML meta-analysis, the effect on all treatments was compared using a hierarchical contrast structure where, as a fixed model, the mortality on blackberry and raspberry populations were compared, followed by a comparison between populations within blackberry ('Tupy' and '2-UDV') and within raspberry ('7-UDV' and '8-UDV'), and its interaction with fungal species assessed. All comparisons were made considering the within-experiment error (estimated by the linear mixed model for each experiment) as the random model of the analysis. Mortality in control treatments never exceeded 14% and none of the cadavers sporulated confirming that no cross contamination had occurred; for this reason control mortality was excluded from the analyses. All analysis was done using GenStat v.8 (Payne et al., 2005).

1.3 Results

1.3.1 Phylogeny reconstruction

The phylogenetic analyses clearly identified all sequences as *T. urticae* with bootstrap values above 80 (Fig. 1). All sequences were grouped with the GF *T. urticae* sample, with an 82% bootstrap value, but separated from the RF *T. urticae* sample with a 99% bootstrap value. Molecular identification was confirmed by morphological identification. Haplotype network analysis based on COI sequences revealed the existence of eight haplotypes, all connected in one large network (Fig. 2). The most abundant was haplotype 1 (H1) with 69 sequences, and haplotype 4 (H4) with four sequences. All other haplotypes contained only one sequence.

Most haplotypes were connected by one mutational change, except for H1 and H4; and H4 and H7, which were both connected by two mutational changes (Fig. 2). AMOVA analysis showed that host plant had no effect on genetic variation, with only 6.53% of the variation being explained by the variety of host plant from which the mites originated. The majority of the genetic variation (93.54%) was due to other, unknown, factors (Table 3).

1.3.2 Susceptibility of T. urticae to fungal infection

Overall, a significant effect of the original host plant species was observed in relation to fungus-induced mortality of adult *T. urticae* females ($F_{1,11.6}$=5.73, P=0.035); mites originally collected from blackberry were more susceptible to infection than those originally collected from raspberry, with the proportions dying being 0.33±0.013 and 0.27±0.018, respectively (Fig. 3). When the effect of fungal isolate was compared, significant effects were also found ($F_{3,73.7}$=23.22, P<0.001); the greatest mortality was achieved using the *M. anisopliae* isolate, followed by *B. bassiana*, then *I. fumosorosea* and finally *L. lecanii* with the lowest mortality (Fig. 2). A significant interaction was found between host plant species and fungal isolate ($F_{3,73.0}$=4.25, P=0.008); the greatest effect of the fungi, particularly *M. anisopliae* and *B. bassiana*, was observed in the blackberry-derived populations compared with the raspberry-derived populations (Fig. 2). No differences were observed between the two populations derived from raspberry (7-UDV and 8-UDV) ($F_{1,49.0}$=0.46, P=0.499), and this was regardless of fungal isolate ($F_{3,49.0}$=0.56, P=0.643). However, there were significant differences between the two populations derived from blackberry ('Tupy' and '2-UDV') ($F_{1,49.0}$=13.75, P<0.001); mites from the '2-UDV' population were more susceptible to

infection than the mites from the 'Tupy' populations (Fig. 2), and this was regardless of isolate ($F_{3,49.5}$=0.91, P=0.443).

1.4 Discussion

The aim of our study was to determine whether the population genetic structure of different *T. urticae* populations could be explained by geographical origin or host plant species, and whether there was an interaction between this genetic variation and their susceptibility to fungal infection. To the best of our knowledge, this is the first report of the relationship between population genetic variation and susceptibility to fungal infection in *T. urticae*. After analysing all our COI sequences, there was genetic variation but it could not be explained by any of the factors we assessed, as demonstrated by the AMOVA analysis.

The haplotype network analysis showed only eight haplotypes. Interestingly raspberry sampling site 7 (Table 2), contained five of the eight haplotypes found in all sequences. This made mites from this sampling site the most genetically diverse as mites from the other sampling sites were from either one or, in some cases, two haplotypes. For this reason the raspberry sampling site 7 population was selected for evaluation in the fungal infection experiment.

It is not clear why this particular population was more genetically diverse, as other populations from the same host plant and even the same variety (7-UDV) did not show the same variation. It is very likely that the control strategy used for this particular orchard may account for this result. Although most producers uses chemicals to control *T. urticae,* the frequency and type of acaricides used can differ greatly amongst orchards; this has been

shown to result in genetic variation in *T. urticae* populations from mandarin orchards (Pascual-Ruiz *et al*., 2014).

Our study confirms previous studies that found very few relationships between host plant and genetic variation (Navajas, 1998; Ros and Breeuwer, 2007). Significant effects of geographical separation on genetic variation have been reported (Xie et al., 2006; Carbonnelle et al., 2007). In our case, the distance between our sampling sites varied from 50 to 2,672 km, which ensured that each population had developed independently. Frequent use of acaricides in citrus orchards has also been associated with a reduction in genetic variation within *T. urticae* populations (Sabater-Muñoz et al., 2012); the suggestion is that only resistant genotypes remain encouraging endogamy (Hada et al., 2016), and leading to reduced genetic variation. Pascual-Ruiz et al., (2014) found greater genetic variation within *T. urticae* populations in citrus under Integrated Pest Management (IPM) than under organic production.

The most genetically diverse population (five haplotypes) originated on the raspberry variety '7-UDV'. The population from the raspberry variety '8-UDV' only had one haplotype, but despite their genetic differences, the susceptibility of these two populations to fungal infection was very similar for all isolates used (Fig 3). In contrast, populations from the two blackberry varieties studied showed very limited genetic variation, with two ('Tupy') and one haplotype ('2-UDV') respectively, but they differed greatly in their susceptibility to fungal infection; the population that originated on '2-UDV' was more susceptible than the population that originated on 'Tupy' (Fig 3). It is clear from our results that the key factor affecting susceptibility to infection was the host plant. Overall, populations from blackberry

were more susceptible to fungal infection, especially *M. anisopliae* and *B. bassiana*, than populations from raspberry.

Variation in virulence of fungal isolates from the same species against *T. urticae* adults has been reported previously (Chandler et al., 2005; Shi and Feng, 2009; Bugeme et al., 2009, 2014; Shin et al., 2017). However, very little has been reported about the effect of host plant on the susceptibility of *T. urticae* to fungal infection (Wekesa et al., 2011). Phenolic compounds, of which gallic acid is the most important, are one of the main metabolites produced by blackberries (Sellappan et al., 2002). Interestingly, gallic acid extracted from the hairy roots of tomato plants, is known to affect the survival of *Helicoverpa armigera* Hübner and *Spodoptera litura* Fabricius (Singh et al., 2014). We believe that gallic acid could affect mite fitness, making them more susceptible to fungal infection. This supports qualitative field observations made by the first author, who recorded that raspberry orchards supported larger populations of *T. urticae* than blackberry orchards, although this requires quantitative confirmation. We cannot find any reports on the gallic acid content of raspberry, but this definitely warrants further research in order to fully understand the tritrophic interactions between host plant, *T. urticae* and entomopathogenic fungi.

In conclusion, host plant was more important than genetic diversity or geographical origin on the susceptibility of *T. urticae* populations to fungal infection; blackberry-derived populations of *T. urticae* were more susceptible to fungal infection than raspberry-derived populations. Our results suggest that plant metabolites could be playing an important role in the outcomes of our experiment, by affecting the fitness of *T. urticae* populations in the field.

With more information about these tritrophic interactions we will be able to design more effective biological control programmes.

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Tables

Table 1: Sampling sites and haplotypes found in the *Tetranychus urticae* populations. * = populations selected for the fungal susceptibility experiments. GC = Geographical coordinates. UDV= Undisclosed variety.

Plant host	Locality	Site number-variety	GC	Sample ID	Haplotype	GenBank
Rubus ulmifolius	Los Reyes, Michoacan	1-UDV	19°34'24.60"N 102°25'32.68"W	44FI	H1	MH285704
				46FI	H4	MH285705
				47FI	H1	MH285706
				49FI	H1	MH285707
				50FI	H1	MH285708
				98FI	H1	MH285727
				99FI	H1	MH285775
				100FI	H1	MH285728
				101FI	H1	MH285729
				102FI	H6	MH285730
				106FI	H1	MH285731
				151FI	H1	MH285732
		2-UDV*	19°34'18.31"N 102°25'36.70"W	37FI	H1	MH285697
				38FI	H1	MH285698
				39FI	H1	MH285699
				40FI	H1	MH285700
				41FI	H1	MH285701
				42FI	H1	MH285702
				43FI	H1	MH285703
				94FI	H1	MH285723
				95FI	H1	MH285724
				96FI	H1	MH285725
				97FI	H1	MH285726
		3-UDV	19°56'3.36"N 102°17'14.83"W	60FI	H1	MH285709
				61FI	H1	MH285710
				62FI	H4	MH285711
				66FI	H1	MH285712
				92FI	H1	MH285721
				93FI	H1	MH285722
		4-Tupy*	19°35'53.14"N 102°30'45.38"W	67FI	H1	MH285713
				68FI	H5	MH285714
				69FI	H1	MH285715
				71FI	H1	MH285716

		5-UDV	19°35'44.76"N 102°31'24.79"W	73FI	H1	MH285717
				74FI	H1	MH285718
				90FI	H1	MH285719
				91FI	H1	MH285720
	Cuauhtemoc, Colima	6-Tupy	19°23'44.37"N 103°36'2.67"W	20FI	H1	MH285756
				21FI	H1	MH285757
				22FI	H1	MH285758
				23FI	H1	MH285759
				24FI	H1	MH285760
				25FI	H1	MH285761
				26FI	H1	MH285762
				27FI	H1	MH285763
				111FI	H1	MH285764
				114FI	H1	MH285765
Rubus idaeus	San Isidro Mazatepec, Jalisco	7-UDV*	20°30'55.82"N 103°39'16.20"W	1FI	H1	MH285733
				2FI	H1	MH285734
				3FI	H2	MH285735
				4FI	H1	MH285736
				5FI	H1	MH285737
				13FI	H1	MH285738
				122FI	H4	MH285739
				125FI	H4	MH285740
				126FI	H7	MH285741
				129FI	H8	MH285742
	Gomez Farias, Jalisco	8-UDV*	19°49'38.02"N 103°28'45.53"W	28FI	H1	MH285743
				30FI	H1	MH285744
				31FI	H1	MH285745
				32FI	H1	MH285746
				33FI	H1	MH285747
				34FI	H1	MH285748
				35FI	H1	MH285749
				36FI	H1	MH285750
				150FI	H1	MH285751
	Jocotepec, Jalisco	9-UDV	20°21'39.86"N 103°31'36.41"W	57FI	H1	MH285752
				85FI	H1	MH285753
				87FI	H1	MH285754
				88FI	H1	MH285755
	San Quintín, Baja California	10-UDV	30°33'54.72"N 115°58'28.32"W	202FI	H1	MH285766
				204FI	H1	MH285767
				208FI	H1	MH285768
				209FI	H1	MH285769
				212FI	H1	MH285770
				214FI	H1	MH285771
				215FI	H1	MH285772

231FI	H3	MH285773
251FI	H1	MH285774

Table 2: Isolates used for the susceptibility experiments. All isolates are held in the Entomopathogenic Fungi Collection at the Colegio de Postgraduados, Mexico. The ATCC isolate was provided by the American Type Culture Collection, Manassas, VA.

Isolate	Species	Host	Origin
Bb88	*Beauveria bassiana*	*Hypothenemus hampei*	Oaxaca, México
Ma129	*Metarhizium anisopliae*	*Tetranychus urticae*	Colima, México
Pfr4	*Isaria fumosorosea*	*Bemisia* sp.	Colima, México
ATTC2009	*Lecanicillium lecanii*	*Toxoptera citricida*	Tucuman, Argentina

Table 3: Results of the analyses of molecular variance (AMOVA) done on COI sequences from *Tetranychus urticae* populations. All tests were based on both molecular distances and haplotypes frequencies.

Source of variation	d.f.	Sum of squares	Variance components	Variation (%)	*F*	*P*
Amongst groups	1	0.258	-0.00013	-0.07	-0.00074	0.46725
Among populations within groups	8	1.990	0.01133	6.53	0.06530	0.04985
Within populations	69	11.194	0.16224	93.54	0.06460	0.05279
Total	78		13.443	0.17344		

Figures

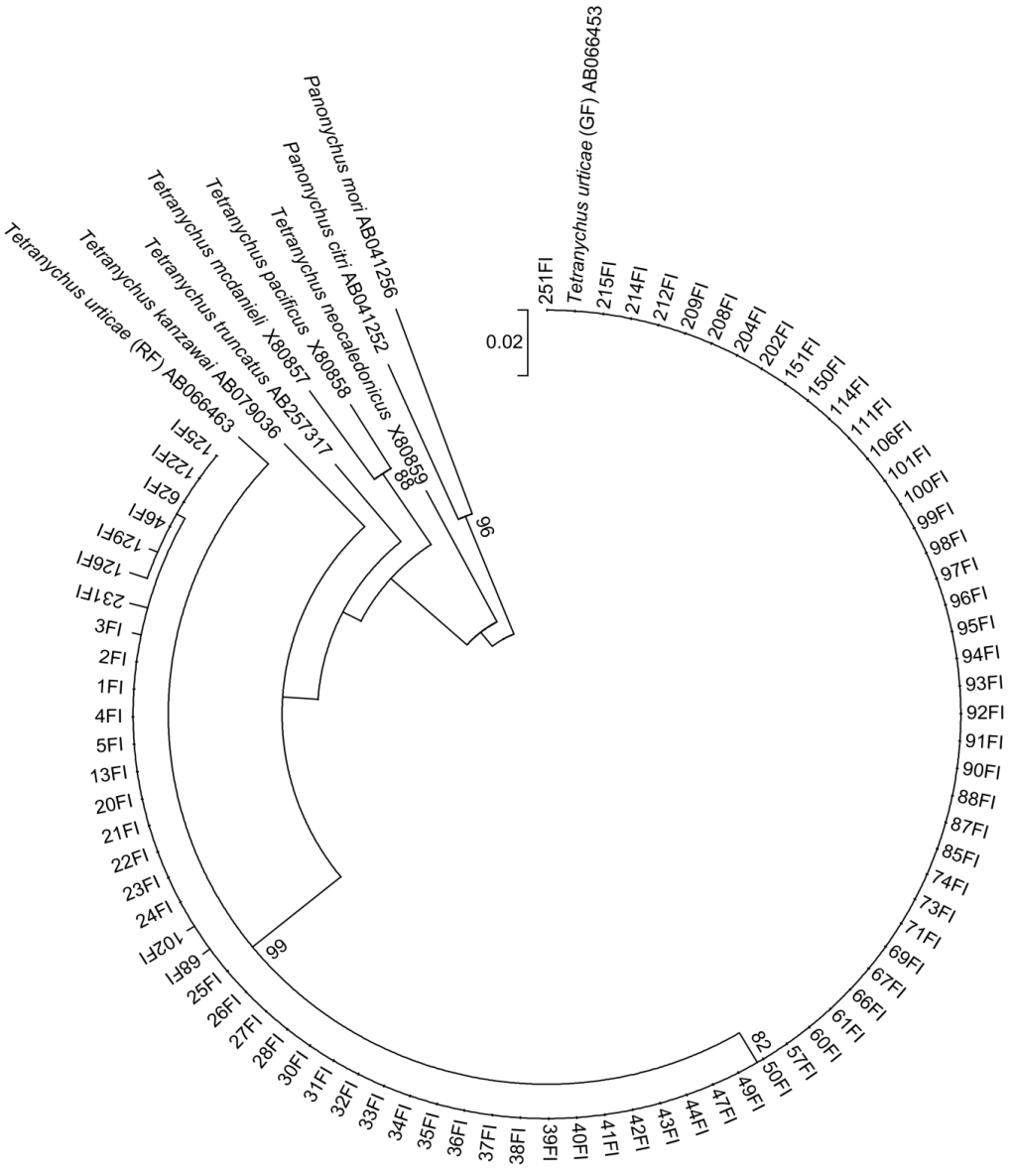

Figure 1: Dendrogram inferred from Maximum likelihood analyses (1,000 replicates) of COI sequences from different *T. urticae* populations. GenBank accession numbers and associated species names are given. Two sequences from *Panonychus* species were used as outgroups. Only bootstrap values above 80 % are shown. Bar depicts the branch length corresponding to 2% ML distance.

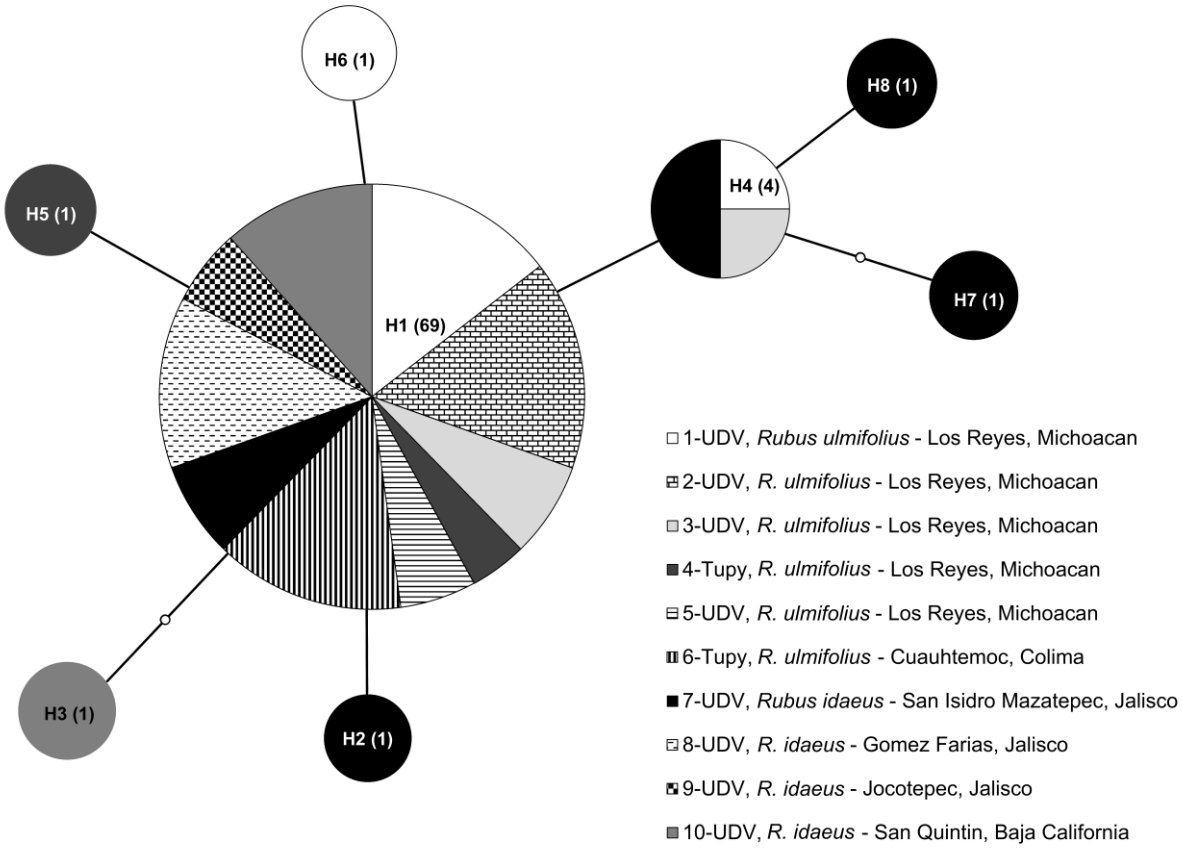

Figure 2: Most parsimonious haplotype network for *Tetranychus urticae* populations. Haplotypes are connected with a 95% confidence limit. Each line in the network represents a single mutational change. Small circles indicate missing haplotypes. Numbers of samples per haplotype are shown in parentheses.

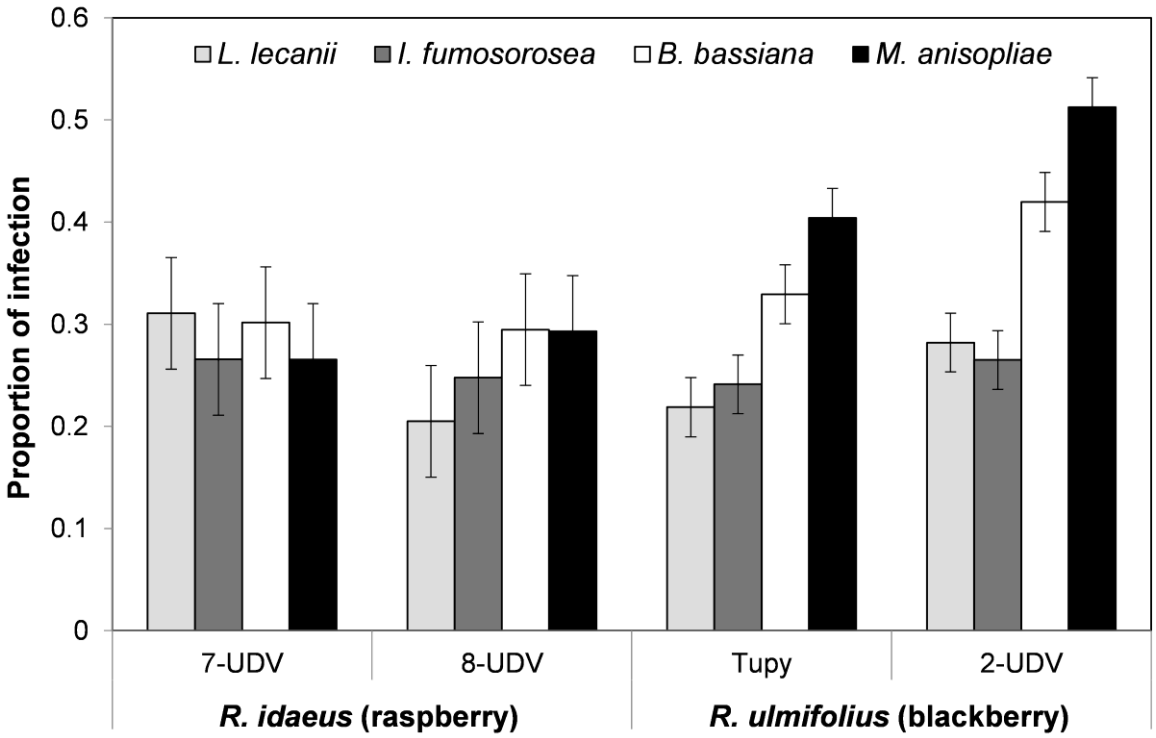

Figure 3: Proportion of adult *Tetranychus urticae* originating from two different host plants (Raspberry var. '7-UDV' or '8-UDV' and Blackberry var. 'Tupy' or '2-UDV') that were killed by four fungal isolates. Error bars represent ± 1x SEM.

DISCUSIÓN GENERAL

El objetivo de este estudio fue determinar si el origen geográfico o especie de planta hospedera de las diferentes poblaciones de *T. urticae* tienen un efecto en la estructura genética poblacional, y si dicha variación genética interactúa con la susceptibilidad a la infección por hongos. Después de analizar todas las secuencias de COI, se encontró una variación genética pero no se pudo explicar por ninguno de los factores que se evaluaron, como lo demuestra el análisis de AMOVA. El análisis de red de haplotipos mostró sólo ocho haplotipos. Curiosamente, el sitio de muestreo 7 de frambuesa (Tabla 2) contenía cinco de los ocho haplotipos encontrados en todas las secuencias, lo que hace que los ácaros de este sitio de muestreo sean los genéticamente más diversos en comparación con otros sitios de muestreo, con sólo uno o en algunos casos dos haplotipos; por esta razón, esta población fue seleccionada para los experimentos de infección fúngica. No está claro por qué esta población en particular mostró esta mayor variación genética, ya que otras poblaciones de la misma planta huésped e incluso la misma variedad (7-UDV) no mostraron la misma variación. Es muy probable que la estrategia de control utilizada para este huerto en particular pudo haber producido este resultado, aunque la mayoría de los productores utiliza control químico, la frecuencia y el tipo de acaricidas utilizados pueden diferir mucho entre los huertos, y esto ha confirmado previamente que produce variación genética en poblaciones de *T. urticae* en huertos de mandarina (Pascual-Ruiz *et al*., 2014). Investigadores anteriores que estudiaron la posible relación entre la planta huésped y la variación genética también encontraron muy poca relación (Navajas, 1998; Ros y Breeuwer, 2007). La variación genética basada en separaciones geográficas también se ha estudiado con efectos significativos (Xie *et al*., 2006; Carbonnelle *et al*., 2007). En nuestro caso, la distancia entre nuestros sitios de muestreo varió

de 50 a 2,672 km, lo que aseguró que cada población se desarrollara de manera independiente. Hada *et al*. (2016) mencionan que la variación genética también puede verse afectada por el número de muestras procesadas, pues a mayor número, mayor será la precisión. Se ha reportado que un uso frecuente de acaricidas en los huertos de cítricos puede conducir a una reducción en la variación genética en poblaciones de *T. urticae* (Sabater-Muñoz *et al*., 2012); permaneciendo sólo los genotipos resistentes que fomentan la endogamia (Hada *et al*., 2016), lo que lleva a una variación genética reducida. Por otro lado, Pascual-Ruiz *et al*. (2014) encontraron una mayor variación genética en poblaciones de *T. urticae* en cítricos bajo la estrategia MIP, la cual incluye el uso de acaricidas, en comparación con el manejo de producción orgánico. Esta variación probablemente esté relacionada con las dinámicas poblacionales naturales, es decir que poblaciones se elevan en condiciones favorables (aumentando la variación genética), pero disminuyen cuando las condiciones no son favorables (reducción en la variación genética) (Aguilar-Fenollosa *et al*., 2011 y 2012). La población más diversa genéticamente (cinco haplotipos) fue la variedad de frambuesa "7-UDV" en comparación con la variedad "8-UDV" de la misma especie hospedera, con un haplotipo; a pesar de esta diferencia genética, su susceptibilidad a la infección fúngica fue muy similar a todos los aislamientos utilizados (Fig. 3). Por otro lado, las dos variedades de zarzamoras estudiadas, "Tupy" y "2-UDV" mostraron variación genética muy limitada, con dos y un haplotipo respectivamente, pero difirieron mucho en su susceptibilidad a la infección por hongos, con la población "2-UDV" como la más susceptible (Fig 3). Es probable que las poblaciones con menor variación genética serán más drásticas al efecto de la aplicación de hongos entomopatógenos y por contrario que las poblaciones con mayor variación genética serán menos susceptibles, (Pascual-Ruiz *et al*., 2014), cómo fue el caso de "7-UDV". De estos

resultados se desprende que el efecto principal sobre la susceptibilidad frente a la infección fue la planta huésped, donde, en general, las poblaciones de zarzamora fueron más susceptibles a la infección por hongos, especialmente contra *M. anisopliae* y *B. bassiana*. La infección de aislamientos de las mismas especies de hongos en adultos de *T. urticae* se ha reportado previamente (Chandler *et al*., 2005; Shi y Feng, 2009; Bugeme *et al*., 2009, 2014; Shin *et al*., 2017). Sin embargo, se ha hecho muy poco por entender el efecto de la planta huésped sobre la susceptibilidad de *T. urticae* a la infección por hongos (Wekesa *et al*., 2011). Uno de los principales metabolitos producidos por las zarzamoras son los compuestos fenólicos, uno de los más importantes es el ácido gálico (Sellappan *et al*., 2002), y curiosamente, el ácido gálico, extraído de las raíces de plantas de tomate, se ha encontrado previamente afectando la supervivencia de *Helicoverpa armigera* Hübner y *Spodoptera litura* Fabricius (Singh *et al*., 2014). Con base a estos resultados, se cree que este compuesto fenólico puede estar afectando la aptitud de los ácaros haciéndolos más susceptibles a la infección por hongos. Esto podría ser parcialmente confirmado por observaciones de campo hechas de manera personal, donde poblaciones más abundantes de *T. urticae* pueden ser observadas en frambuesa que en huertos de zarzamora, aunque esto requiere de confirmación cuantitativa. No se encontraron informes sobre el contenido de ácido gálico en la frambuesa, pero esto definitivamente requiere más investigación para comprender completamente las interacciones tritróficas entre la planta huésped, *T. urticae* y los hongos entomopatógenos.

En conclusión, con los resultados del presente estudio, se registró que la planta huésped tuvo un efecto significativo en comparación con la diversidad genética o el origen geográfico en la susceptibilidad de las poblaciones de *T. urticae* a la infección por hongos, como lo demuestra la mayor susceptibilidad de las poblaciones de zarzamora en comparación

con las de frambuesa. Estos resultados sugieren que los metabolitos de las plantas podrían desempeñar un papel importante en nuestros resultados, al afectar la aptitud de las poblaciones de *T. urticae* en el campo. Se podrán diseñar programas de control biológico más efectivo, en cuanto más información se genere sobre estas interacciones tritróficas.

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