El Colegio de la Frontera Sur

Diversidad genética de *Metarhizium anisopliae* en cañaverales de Tabasco y su virulencia sobre *Aeneolamia postica*

TESIS
presentada como requisito parcial para optar al grado de
Doctora en Ciencias en Ecología y Desarrollo Sustentable

por

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2013

Anexo 23

El Colegio de la Frontera Sur

Tapachula, Chiapas, 21 de Noviembre de 2012.

Las personas abajo firmantes, miembros del jurado examinador de: **Arely Bautista Gálvez** hacemos constar que hemos revisado y aprobado la tesis titulada Diversidad genética de *Metarhizium anisopliae* en cañaverales de Tabasco y su virulencia sobre *Aeneolamia postica*. Para obtener el grado de **Doctor (a) en Ciencias en Ecología y Desarrollo Sustentable**

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DEDICO ESTA TESIS

A MI ESPOSO E HIJO

A mi esposo Julio Cesar Almaraz Navarro por todo su apoyo, compresión y amor durante mis estudios de Doctorado.

A mi hijo Julio Cesar Jr. porque el día que lo trajimos al mundo me di cuenta que Dios no ha perdido la esperanza en nosotros los seres humanos, además que mi hijo ha sido la motivación para la culminación de este proyecto.

Agradecimientos

Agradecerle a mi tutor al Dr. Juan Francisco Barrera Gaytán por sus asesorías, consejos en mi formación profesional de mis estudios de Doctorado y todo su apoyo brindado para la culminación de este proyecto.

Al Dr. Juan Florencio Gómez Leyva por su amistad brindada y apoyo en asesorías en la parte de la caracterización molecular.

Al Mtro. Javier Valle Mora por todo su apoyo brindado en los análisis estadísticos.

A mis asesores:
Dr. Jaime Gómez Gómez por su amistad y asesorías brindadas
Dr. Emeterio Payró de la Cruz por su amistad brindada y su apoyo en la parte molecular
Dr. Sergio Salgado García por su amistad y asesorías brindadas en este trabajo

Durante mi estancia en ECOSUR-Unidad Tapachula recibí apoyo de varias personas por lo que agradezco profundamente: A la Familia Bustamante y especial a Doña Beny por su hospitalidad brindada durante mis estudios de Doctorado, a mi amiguita Mayra Martínez Solís por su apoyo incondicional y a mi amigo Jorge Cancino por haber compartido la mayoría de los cursos juntos por nuestra orientación en Plagas.

A Rosalba Morales por su apoyo técnico durante mi estancia en ECOSUR, Ana María Galindo y Margarita Hernández por su cooperación en la adquisición de material bibliográfico y Beatriz Romero por su apoyo técnico

Al I.Q.A. Jesús Manuel Llergo Asmitia rector de la Universidad Tecnológica del Usumacinta (2003-2011) por haberme otorgado el permiso como docente para realizar mis estudios de Doctorado.

Al Ing. Alberto Lastra González rector de la Universidad Tecnológica del Usumacinta (2012--) por su apoyo en la gestión de este proyecto con los productores cañeros de la región y por su amistad brindada.

Al Mtro. Roberto Sosa Rincón por su amistad y apoyo brindado para la culminación del Doctorado

Agradezco al Consejo Nacional de Ciencia y Tecnología (CONACyT) por la beca que me fue otorgada para realizar mis estudios de Doctorado.

Fondos-Mixtos CONACYT-Tabasco (Proyecto TAB-2007-C09-74101) por haber financiado este trabajo de investigación.

Agradezco al Programa del Profesorado (PROMEP) por el apoyo que me fue otorgado para realizar mis estudios de Doctorado

CONTENIDO

ABSTRACT

Metarhizium anisopliae (Metschnikoff.) Sorokin (Moniliales: Moniliaceae) is an entomopathogenic fungus used as a biological control agent against the sugarcane froghopper *Aeneolamia postica* (Wlk.) (Hemiptera: Cercopidae), one of the main pests of sugarcane in Tabasco, Mexico. Since 2005, multiple isolates of *M. anisopliae* have been applied by farmers in crop areas; however, the current genetic diversity of the fungus population in the field is unknown. The aim of this study was to characterise isolates of *M. anisopliae* collected in sugarcane fields to better understand their genetic and virulence characteristics against *Aeneolamia postica*. In particular, the impact of introduced isolates on the genetic diversity of native strains was determined. Sampling of mycosed insects was conducted in soils from the main sugarcane fields of Tabasco. Twenty isolates of *M. anisopliae* were obtained and a monosporic culture from each one was used for the characterisation studies. The isolates were characterised using both morphological characteristics and rDNA analysis. The genetic diversity of the fungus was analysed by means of RAPD molecular markers. One of the isolates was significantly similar to the isolate introduced by farmers. RAPD analysis revealed a homogeneous group among the native isolates collected in sugarcane fields in Tabasco. These results suggest a very low incidence of recombination between native and introduced isolates. The implications of the findings on the genetic diversity of *M. anisopliae* and the use of this pathogen for biological control products for *Aeneolamia postica* are discussed in this paper.

Palabras clave: molecular markers, RAPD, patogenicity, biological control

CAPÍTULO I
INTRODUCCIÓN

CAPÍTULO I

INTRODUCCIÓN

La caña de azúcar (*Saccharum* spp.) dio origen a un sistema agroindustrial que ocupa un lugar preponderante y trascendente en la actividad económica y social de México, de tal manera que es la agroindustria más importante. Durante la zafra 2011/2012, se cultivaron 703,761 mil hectáreas de caña de azúcar en 15 estados de la República, en los cuales se ubican 54 ingenios que operan con un rendimiento promedio en campo de 71 ton ha^{-1} y de fábrica de 10.92 % y México se mantiene dentro de los 10 principales países productores de azúcar en el mundo y tiene un consumo per cápita de 40 kg, ubicándose así en el grupo de países de mayor consumo de azúcar. Se estima que más 3 millones de mexicanos dependen de esta actividad económica, la cual es una importante generadora de divisas (CNPR, 2012).

Sin embargo, la producción y calidad de la caña de azúcar se ve limitada, los costos de producción se elevan y el impacto negativo al ambiente se incrementa por la aplicaciones de productos químicos destinados al control de sus principales plagas, siendo la mosca pinta o salivazo (*Aeneolamia postica*) la más perjudicial en extensas áreas en los litorales del golfo de México y del océano Pacífico. Esta plaga provoca reducciones de hasta el 60% en los rendimientos de la caña de azúcar, por lo que el productor se ve obligando a realizar de 3 a 4 aplicaciones de productos químicos en promedio durante el periodo de lluvia. Si bien este control crea buenos resultados en las primeras aplicaciones, con el tiempo los insectos crean resistencia, elevando los costos de producción al incrementarse el uso de productos químicos para lograr resultados similares a los obtenidos inicialmente.

La solución a la contaminación ambiental no es sencilla, pues la población mundial en su acelerado crecimiento geométrico demanda la producción de más alimentos en este sentido, se estima que en los próximos 25 años aumentará en un 72 por ciento y es de esperar que, para entonces, se habrá reducido el déficit alimentario, aumentado el consumo

de alimentos per cápita en los países que padecen escasez y se diversifiquen los regímenes alimenticios de las poblaciones, todos estos cambios tendrán un gran peso en los sistemas de producción de alimentos, en los recursos naturales y en el medioambiente (FAO, 2008).

Por otra parte, los hongos entomopatógenos, microorganismos asociados a insectos que habitan diversos ambientes, incluyendo agroecosistemas, han sido utilizados en el control biológico de plagas como alternativa al uso de productos químicos o como parte del manejo integrado de plagas (Zimmermann, 2007). El potencial para "matar plagas" por parte de los entomopatógenos, llamado comúnmente patogenicidad, está determinado por las características genéticas del individuo (Robertson *et al*., 2007).

Los estudios moleculares hoy en día resultan ser una herramienta de suma importancia en la caracterización de aislamientos de hongos entomopatógenos y para el conocimiento de su variabilidad genética, sin embargo la investigación sobre estos microorganismos se ha limitado a la entomología aplicada y a las consideraciones ecológicas relacionadas con el manejo integrado de plagas. Hasta 1980, poca información sobre la variación genética en patogenicidad era conocida por los genetistas de los hongos entomapatógenos (Hajek y St. Leger, 1994), con el advenimiento de técnicas bioquímicas y moleculares fue rápidamente expandido para conocer la variabilidad intraespecifica de los hongos por medio de marcadores moleculares, por lo que recientemente se están haciendo esfuerzos para estudiar la genética de estos hongos.

Los marcadores moleculares de ADN han demostrado ser muy valiosos principalmente en estudios de la diversidad genética y mapeo de genes estas técnicas han tomado auge desde el desarrollo de la reacción en cadena de la polimerasa (PCR) (por sus siglas en inglés Polymerase Chain Reaction). Entre las técnicas más comunes en este campo pueden mencionarse los RFLPs (por sus siglas en inglés, Restriction Fragment Length Polymorphism), AFLPs (por sus siglas en inglés, Amplified Fragment Length Polymorphism), RAPD (por sus siglas en inglés, Randomly Amplified Polymorphic DNA), entre otros (Whitkus *et al.*, 1994). Estas técnicas resultan ser herramientas que facilitan la identificación mediante la detección de polimorfismo y el desarrollo de perfiles genéticos entre los aislamientos, razas, subespecies de interés. La metodología mayormente usada en

estudios de diversidad genética han sido los RAPDs, técnica basada en PCR (Williams *et al*., 1990). También estos marcadores han sido utilizados para asociar patrones específicos a índices de patogenicidad (Gaitán *et al*., 2002). Los RAPD pueden ser útiles para rastrear cepas entomopatogenas específicas de *Metarhizium anisopliae* (Hegedus *et al.* 1996; Fegan *et al.*1993).

Los RAPD basados en la incorporación de desoxinucleotidos fluorescentes proveen una herramienta útil para la identificación de hongos entomopatógenos y esta técnica es especialmente aplicable para el screening de muchos aislados en el estudio de poblaciones (Bidochka, 1994). Esta técnica ofrece ventajas: la cantidad de ADN es reducida (10-25ng), los partidores son pequeños, no se requiere de un conocimiento previo a la genética del organismo y la ejecución es de poca complejidad y de menor costo que otras técnicas (Williams *et al.,* 1990). Los RAPDs han sido ampliamente utilizados para caracterizaciones moleculares y análisis de plantas, bacterias y especies de hongos (Cobb y Clarkson, 1993). Sin embargo, hoy en día han sido aplicados para *Metarhizium anisopliae* debido al potencial uso de este hongo como agente de control biológico, se han desarrollado numerosas y variadas investigaciones entre las que destacan aquellas relativas a la determinación de cepas (Yip *et al.* 1992, Bidochka *et al.,* 2005; Fegan *et al.,* 1993; Cobb y Clarkson 1994; Guerrero, *et al.* 2000; Kedall y Rygiewicz, 2005), para detectar las variaciones entre aislamientos (Freed, *et al*., 2011). La importancia de estos análisis radica en la exactitud de la identificación de los entomopatógenos, permitiendo contar con aislados más puros.

En esta investigación, se planteó como objetivo estudiar la diversidad genética de *Metarhizium anisopliae* mediante la amplificación de segmentos de ADN usando RAPD por sus siglas en inglés (Random Amplified Polymorphic DNA). Este conocimiento genético básico permitirá su manipulación para la obtención de cepas para la caracterización de los ceparios, así como para la producción de determinados biopreparados

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OBJETIVOS

OBJETIVO GENERAL

Estudiar la diversidad genética de *Metarhizium anisopliae* mediante la amplificación de segmentos de ADN usando partidores de diseño aleatorio (Random Amplified Polymorphic DNA, RAPD) y probar su virulencia en la mosca pinta (*Aeneolamia postica*).

OBJETIVOS ESPECÍFICOS

1. Colectar *M. anisopliae* en cañaverales de la región de los Ríos del estado de Tabasco.

2. Aislar *M. anisopliae* a partir de larvas de *Galleria mellonella* expuestas a muestras de suelo y de insectos micosados colectados en campo.

3. Caracterizar morfológicamente aislamientos de *M. anisopliae*

4. Caracterizar genéticamente aislamientos de *M. anisopliae* con marcadores moleculares.

5. Determinar la virulencia de los aislamientos de *M. anisopliae* genéticamente caracterizados sobre *Aeneolamia postica* en laboratorio.

6. Conocer la relación entre la variabilidad genética de los aislamientos de *M. anisopliae* y su virulencia sobre *Aeneolamia postica*.

CAPÍTULO II

MORPHOLOGY AND PRODUCTION OF CONIDIA FROM *Metarhizium anisopliae* ISOLATES COLLECTED IN CHIAPAS,TABASCO AND VERACRUZ, MEXICO.

MORPHOLOGY AND PRODUCTION OF CONIDIA FROM *Metarhizium anisopliae* ISOLATES COLLECTED IN CHIAPAS,TABASCO AND VERACRUZ, MEXICO.

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RESUMEN

La caracterización de aislamientos de hongos entomopatógenos proporciona conocimiento básico para la selección de aquellos con mejores características para ser usados en programas de control biológico de plagas. Por lo anterior, el presente trabajo tuvo como objetivo comparar la morfología y producción de conidios de aislamientos de *Metarhizium anisopliae* (Metchnikoff) Sorokin provenientes de Chiapas, Tabasco y Veracruz, México. En total, se compararon 15 aislamientos, de los cuales 1 fue de Chiapas, 10 de Tabasco y 4 de Veracruz. Los aislados se caracterizaron por presentar hifas septadas, conidias cilíndricas-ovoides y el tamaño de las conidias fue 1.802 a 5.422 µm en longitud, con diferencias estadísticas entre los aislamientos (gl= 14, F= 3.45, *P*=0.001), color de la colonia verde oscuro oliva, gris verde claro y oliva amarillo. El porcentaje de germinación a las 24 hrs fluctuó entre 87 a 96 %. El crecimiento radial de los aislamientos de las colonias se caracterizó por un crecimiento circular y fueron separados cuatro grupos por su crecimiento radial fue estadísticamente diferente entre los aislamientos (gl 14, 60; F= 523.02; $P < 0.0001$). La respuesta en crecimiento radial, al igual que la producción de conidios, pigmentación de las colonias permiten determinar aislamientos de *M. anisopliae* para trabajar con control biológico de plagas.

PALABRAS CLAVE: *germinación/ control biológico/ caña de azúcar*

ABSTRACT

Characterization of entomopathogenic fungi isolates provides basic knowledge for the selection of those with the best characteristics for use in biological pest control programs Consequently, the aim of this study is to characterize the morphology and production of

conidia from *Metarhizium anisopliae* (Metchnikoff) Sorokin isolates. A total of 15 isolates were characterized; one from Chiapas (commercial product), 10 from Tabasco and four from Veracruz. The isolates were characterized by the presence of septate hyphae containing cylindrical-ovoid conidia with a length of between 1.802 to 5.422 µm. Differences between isolates were statistically significant (P=0.001). Colony colour varied between dark olive green and yellow olive. After 24 hrs, isolate conidia germination varied between 87% and 96%. The radial growth rate of the colony presented differences between isolates (P < 0.0001). With regard to conidia, no significant differences were found (P>0.05) between isolates 14 days succeeding inoculation. The implications of these results for taxonomic identification and biological control using *M. anisopliae* are discussed.

KEY WORDS: *germination/biological control/sugar cane*

Introduction

In laboratories dedicated to entomopathogenic fungi production, knowledge of the morphological, biochemical and molecular characteristics of the biological material is important as this provides relevant information for the identification and use of these organisms in biological control programs. Regarding *Metarhizium anisopliae* (Metchinikoff) Sorokin, an entomopathogen frequently used against a wide diversity of pest insects, diverse studies have been carried out in order to characterize the isolates of this particular fungi, considering conidia morphology (Yip et al., 1992; Romero et al., 1997; Padilla et al., 2000; Liu et al., 2003), production and colony radial growth (Varela and Morales, 1996; Steven and Matthevew, 2001). Yip et al. (1992), found that in all *M. anisopliae* isolates, conidia morphological characteristics were similar , although some possessed the ability to grow at low temperatures (5ºC). Size of *M. anisopliae* conidia depends on the culture medium used (Kamat et al., 1952). With respect to fungi coloration, Tulloch (1976) suggested that this characteristic only merits secondary taxonomic value. Nevertheless, entomopathogenic fungi identification is still based on morphological criteria (St. Leger et al., 1988). Several studies of morphological characteristics have been carried out with *M. anisopliae* (Padilla et al., 2000; Bidochka and Kamp 2002; Bisshooff et al., 2006; Iskandarov et al., 2006).

In Tabasco, Mexico, several sugar cane farmers have used *M. anisopliae* for the biological control of *Aeneolamia postica*. (Hemiptera: Cercopidae) .Thus a research project was initiated with the aim of generating information to strengthen biological control via the following objectives: i) establish a culture collection of *M. anisopliae* using isolates collected in sugar cane fields of the region; ii) characterize biological diversity of isolates using various techniques; and iii) determine isolate pathogenicity against *Aeneolamia* spp. This study presents the results of the research carried out to characterize colony radial growth as well as morphology and production of conidia from *M. anisopliae* isolates collected in Tabasco. These isolates were compared with those collected in Veracruz and from a *M. anisopliae* commercial product produced in Chiapas and used against *Aeneolamia* postica in Tabasco.

Methods and Materials

This study was developed in the laboratory of the Biotechnology Department of the Universidad Tecnológica del Usumacinta (Technological University of the Usumacinta River) , located in Emiliano Zapata, Tabasco, Mexico.

Origin of Isolates

Fifteen *M. anisopliae* isolates were studied (Table I). Ten isolates were collected from the Los Rios sugar cane cultivation region of Tabasco between 2007 and 2008; four collected in Veracruz were provided by the Centro Nacional de Referencia de Control Biológico (CNRCB) (National Plant Health Centre) located in Tecomán, Colima ; and one provided by the "Tiemelonlá Nich Klum" farmers association laboratory, based in Palenque, Chiapas, which has been commercially applied to combat *Aeneolamia* spp in sugar cane fields of Tabasco.

Regarding *M. anisopliae* isolates collected in Tabasco, two were obtained from *Aeneolamia postica* adults captured in sugar cane fields and eight obtained from *Galleria mellonella L*. (Lepidoptera: Pyralidae) larvae found in sugar cane field soil samples (Vanninen, 1997) . The isolates from Veracruz and the commercial product from Chiapas were obtained from micosed *Aeneolamia postica* adults (Table I). All the isolates were cultivated in Potato Dextrose Agar (PDA) medium at 30 $^{\circ}$C in complete darkness (Bruck

et al., 2005) and 80 % relative humidity. Identification of *M. anisopliae* isolates was carried out using the reproductive structures of the fungi and the keys developed by Tulloch (1976).

Shape and size of conidia.

Conidia morphology was characterized using scanning electron microscope photographs. The fungi samples were processed as follows: Petri dishes containing fungi in the culture medium were exposed to osmium tetroxide at 1 % for 1 h before cutting two 0.3 x 0.6 cm sections of agar with mycelium. These were then submerged in vials containing a fixing solution of 4 % glutaraldehyde dissolved in a buffer solution of 0.1M, pH 7 sodium cacodylate (SBC), with 0.01 M $CaCl_2$ and 1 %. sodium dodecyl sulphate (SDS) at Immediately afterwards a small vacuum was created in the vials to extract the air occluded in the mycelium and ensuring that the pieces of agar remained submerged. After 3 h, the fixing solution was replaced with an identical fresh solution but without SDS and left overnight. The next day the sample was washed for 5 min with filtered distilled water and subsequently post fixed with a solution of 1% osmium tetroxide. Approximately 18h later the osmium tetroxide was removed and the sample was washed with filtered distilled water (two changes every 5 min). Dehydration was carried out by changes in a series of ethanol solutions (30, 50, 70, 90 and 100%) , each lasting 30 min , with a second ethanol change at 100%. Sample drying was undertaken using a SPI Supplies critical point dryer, model SPI-Dry CPD; for drying, the agar pieces were transported in bags made from eyeglass cleaning paper , with a small ring on the inside providing support and preventing any agar pieces from collapsing; the bags were sealed with aluminium clips and dried at the CO_2 critical point. The dried sample was then mounted in aluminium cylinders using extra sticky double-sided Sellotape and the space between the microscopic slide and the sample was filled with silver paint and covered with a 20nm thick gold-palladium layer. Finally, the sample was observed under an electron scanning microscope and at a working distance of 10 mm.

Colony Color

The color of *M. anisopliae* isolates was determined using the methodology established by Watrud et al. (2006), who established the colony color of several species of fungi via a process similar to that used by Munsell (1977) to determine the color of plant tissue and soil.

Conidia Germination

Conidia germination for each isolate of *M. anisopliae* was determined using a solution that was prepared by placing 1g of fungi in sterile distilled water with Tween-20 at 0.03 %. Dilutions of this solution were performed taking 1 ml each time. 50 µl was extracted from the fourth dilution and placed on five points of a microscope slide with a PDA medium and incubated in darkness at 30º C. At 24 h the slide was observed at 40 X with an optical microscope. To detain fungi development, a drop of lactophenol cotton blue was placed on the microscope slide, and subsequently 300 spores were counted, recording germinated and non-germinated spores. In total, three slides were observed for each isolate. The criteria used to determine if a conidia had geminated, was whether conidia size was the same or greater than the germinative tube (Goettel and Inglis, 1997).

Colony Radial Growth

A study was carried out to compare colony radial growth of 15 *M. Anisopliae* isolates. A 0.5 x 0.5 cm 4 day-old fragment from each isolate was placed in the centre of 1.5 x 9.0 cm petri dishes containing a PDA culture medium, subject to a temperature of 30 °C and in darkness. Every 24 hrs, during four days, colony growth was recorded by measuring growth in four equidistant radiuses previously drawn on the underside of each petri dish. The data was analysed by means of a completely random analysis of variance with five repetitions using the SAS ver. 8.02 (1999-2011) statistical programme.

Conidia production.

Each of the 15 *M. anisopliae* isolates were in petri dishes containing a PDA culture medium. Fourteen subsequent to inoculation, a fragment was cut from the colony using an assay tube to obtain a 1cm diameter circle (Kamp and Bidochka, 2002). The fragments were submerged in 2 ml eppendorf tubes, with sterile distilled water and Tween-20 at

0.1% which were shaken vigorously in a vortex for about 1 min. Depending on the isolate, between 4 and 9 dilutions were made to allow counting of the conidia using a microscope with a Neubauer camera (Goettel and Inglis, 1997).

Results and Discussion

Conidia shape and size

The shape and size of the isolate conidia studied are presented in Table II and Figure 1. As can be observed, all the isolates presented cylindrical-ovoid conidia, typical of *M. anisopliae* (Tulloch, 1976; Bridge et al., 1993; Humbert, 1997). The length of the conidia varies from between 1.802 and 5.422 µm, with statistically significant differences between isolates (gl= 14, F= 3.45, *P*=0.001). According to the Tukey key , isolate length in conidia BC0710, L0909 and SF0811 were statistically the same but longer than the conidia found in isolates M370, FC0805, GB0808, AD0702 and FC0706, which also presented statistically similar lengths. Conidia lengths were statistically similar in isolates CD0804, M374, M371, AS0807, M372, MM0801 and AD0803. Although some authors indicate that the size of conidia of *M. anisopliae* depends on the culture medium used (Kamat et al., 1952; Kamp and Bidochka, 2002), a PDA culture medium was used for all the isolates in our study. In this case, conidia size was an intrinsic characteristic of the isolates.

Colony color.

At the initiation of colony growth, all the *M. anisopliae* isolates were white, however as these matured they turned dark green, light olive-green, light green grey and yellow olive, depending on the particular isolate (Table II). The change observed in the fungi colony coloration is consistent with that reported in the literature (Bischoff et al., 2006). With the exception of isolate CD0804, the culture medium of all the isolates was stained pink after 20 days, a characteristic that was evident when observing underneath the petri dish. According to Griffin (1994), this pigment has been associated with spore resistance to abiotic factors and can help differentiate isolates. CD0804 was the only isolate that presented a yellow olive colour. These differences indicate that in certain cases coloration could be a taxonomic characteristic of more importance than suggested by other authors (Tulloch, 1976; Yip et al., 1992).

Conidia germination

All of the *M. anisopliae* isolates germinated 24 h after inoculation; germination percentage fluctuated between 87 and 96 % (Table II). AD0803 was the only isolate that presented less than 90 % germination. Guerrero et al. (1999) reported between 32 and 92% germination in 10 *M. Anisopliae* isolates.

Colony Radial Growth.

The radial growth rate of the *M. anisopliae* colony was statistically different among the isolates (gl= 14, 60; F= 523.02; $P < 0.0001$), four groups were identified, of which the first presented the fastest growth and the fourth the slowest. The first group consisted of isolate GB0808 from Tabasco, displaying a growth rate of 4.5cm at 72 h; the second group, with a growth of 4.5 cm at 96 h, was composed of isolates MM0801, AD0803, CD0804 and AD0702, also originating from Tabasco; the third group, made up of isolates FC0705, FC0706, SF0811, M371 and M374 from Tabasco and Veracruz presented growth of less than 4.5 cm at 96 h; the fourth and final group was characterized by presenting a growth rate of less than 3 cm at 96 h and was formed by isolates AS0807 and BC0710 from Tabasco, M370 and M372 from Veracruz and L0909, a commercial product from Chiapas. Fungi growth rate in a culture medium is indicative of its capacity to grow and compete with other microorganisms such as saprophytes in diverse substrates or pathogens on a hosts cuticle (Liu et al., 2003). The radial growth rate of most isolate colonies collected in Tabasco was higher than the growth rate displayed by L0909, the *M. Anisopliae* commercial product.

Conidia Production

Conidia production of the studied isolates is presented in Table III. No significant differences were found between isolates. Based on morphological and biological characteristics considered in this research, several native isolates of *M. anisopliae* from the Rios region of Tabasco with regard to control of *Aeneolamia potica* With the aim of selecting the isolates with the potential for the biological control of this pest, further research related to laboratory production and effectiveness in the field is required.

Although morphological and biological characterization of entomopathogenic fungi are useful tools for the identification of variability and selection of pathotypes with characteristics favouring survival and efficacy, complementary molecular characterization is also essential. The latter not only allows identify genetic variabilty of the fungi but also establishes the genetic origin of the isolates, thus exploring the relationship between fungi genetic characteristics and pathogenicity (Bautista-Gálvez et al., 2012).

In conclusion, the present study demonstrates that conidia size rather than shape, could be an important morphological characteristic in the differentiation of *M. anisopliae* isolates; with the exception of colony coloration which played a lesser role in isolate identification. biological aspects, radial growth rate of *M. anisopliae* colonies was the most important variable in differentiating isolates, while conidia germination and production did not provide sufficient information for isolate identification.

ACKNOWLEDGMENTS

Thanks to Fondos-Mixtos CONACYT-Tabasco (Project TAB-2007-C09-74101) for financing this project and Javier Valle-Mora (ECOSUR) for his support with the statistical analysis of the data..

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Table I. *Metarhizium anisopliae* isolates used in study

Isolate Code	Insect source of isolate	Origin	Year of collection
MM0801	*Aeneolamia postica*	Tabasco	2008
AD0702	*G. mellonella*	Tabasco	2007
AD0803	*G. mellonella*	Tabasco	2008
CD0804	*G. mellonella*	Tabasco	2008
FC0805	*G. mellonella*	Tabasco	2008
FC0706	*G. mellonella*	Tabasco	2007
AS0807	*G. mellonella*	Tabasco	2008
GB0808	*Aeneolamia p*ostica	Tabasco	2008
L0909[1]	*Aeneolamia postica*	Chiapas	Unknown
BC0710	*G. mellonella*	Tabasco	2007
SF0811	*G. mellonella*	Tabasco	2008
M370[2]	*Aeneolamia postica*	Veracruz	1994
M371[2]	*Aeneolamia postica*	Veracruz	1994
M372[2]	*Aeneolamia postica*	Veracruz	Unknown
M374[2]	*Aeneolamia postica*	Veracruz	1995

[1] *Metarhizium anisopliae* isolate provided by the "Tiemelonlà Nich Klum" laboratory located in Palenque, Chiapas, Mexico. [2] CNRCB = Centro Nacional de Referencia en Control Biológico, Tecoman, Colima, México

Table II. Characteristics of conidia from *Metarhizium anisopliae* isolates, 24 h after culture medium innoculation

Isolate code	Conidia shape	Conidia length (µm) [1]	Color according to Munsell (1977)	Conidia germination (%)
MM0801	Cylindrical- ovoid	2.686 AB	Dark green	92
AD0702	Cylindrical- ovoid	1.874 B	Dark green	93
AD0803	Cylindrical- ovoid	2.230 AB	Dark green	87
CD0804	Cylindrical- ovoid	3.390 AB	Yellow olive	93
FC0805	Cylindrical- ovoid	1.982 B	Dark green	92
FC0706	Cylindrical- ovoid	1.802 B	Dark green	92
AS0807	Cylindrical- ovoid	2.620 AB	Light green	92
GB0808	Cylindrical- ovoid	2.084 B	Dark green	92
L0909	Cylindrical- ovoid	4.680 A	Dark green	93
BC0710	Cylindrical- ovoid	5.422 A	Dark green	95
SF0811	Cylindrical- ovoid	4.814 A	Light olive green	96
M370	Cylindrical- ovoid	2.106 B	Light grey green	91
M371	Cylindrical- ovoid	3.821 AB	Dark green	90
M372	Cylindrical- ovoid	2.450 AB	Dark green	93
M374	Cylindrical- ovoid	2.400 AB	Dark green	93

[1] Values with the same letters are statistically equal as according to the Tukey test ($p<0,05$)

Table III. Mean production of conidia from 15 isolates of *Metahizium anisopliae* in a potato dextrose agar medium (PDA).

Isolate code	Conidia production conidia/ml (x 10^8)	
	Mean	Standard Error
MM0801	4.695	1.698
AD0702	5.99	1.720
AD0803	1.834	0.566
CD0804	2.179	0.631
FC0805	2.361	0.632
FC0706	2.310	1.152
AS0807	-	-
GB0808	3.349	1.146
L0909	3.500	1.052
BC0710	-	-
SF0811	1.007	0.8972
M370	2.775	0.900
M371	1.371	0.903
M372	2.775	1.193
M374	1.088	-

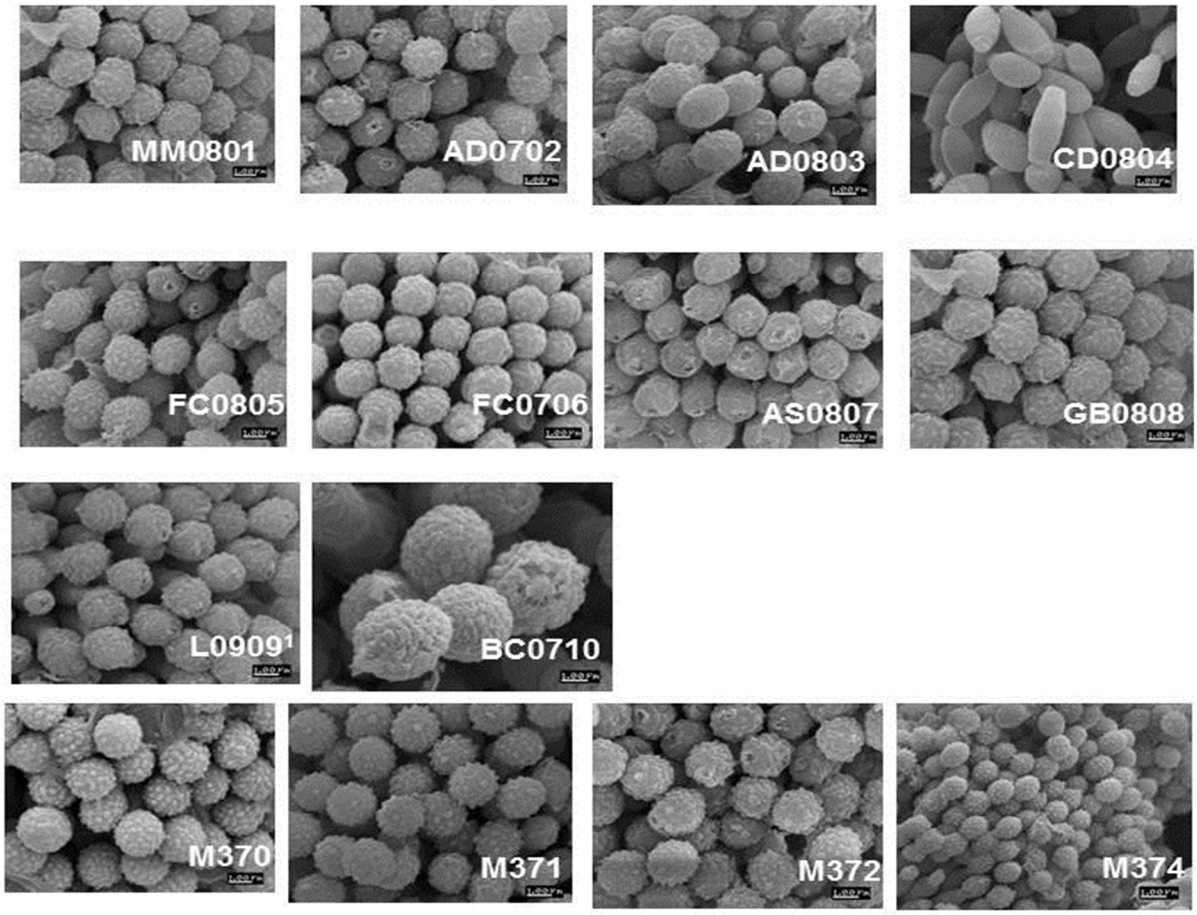
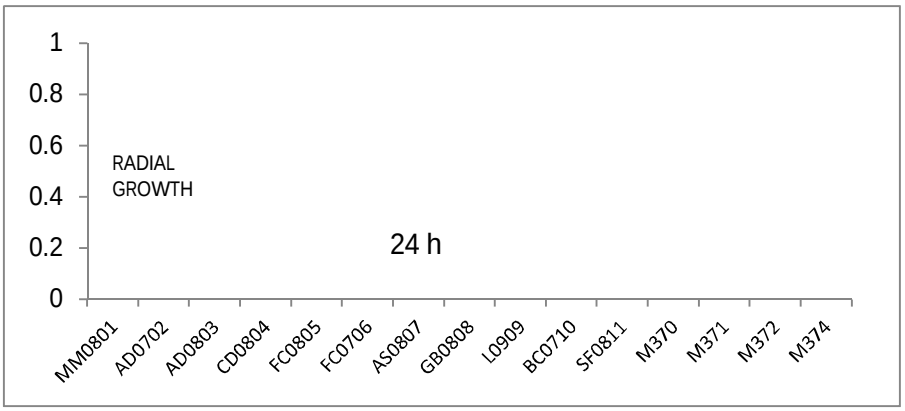


Figure 1. Microscopic picture of the conidium of *Metarhizium anisopliae isolate*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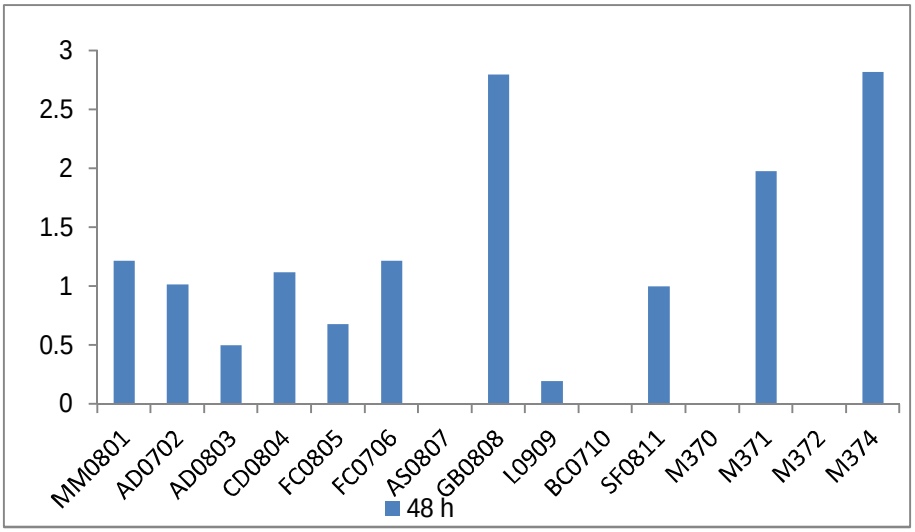
0.8
0.6
0.4
0.2
0
RADIAL
GROWTH
24 h
MM0801
AD0702
AD0803
CD0804
FC0805
FC0706
AS0807
GB0808
L0909
BC0710
SF0811
M370
M371
M372
M374


3
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MM0801
AD0702
AD0803
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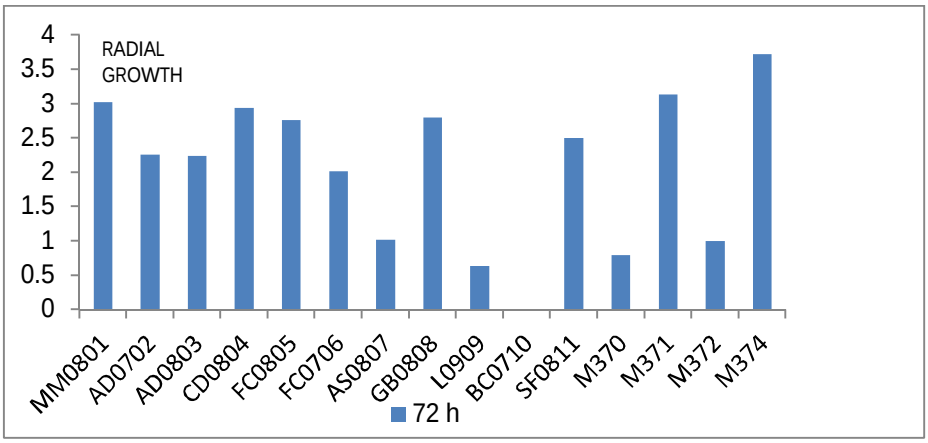
BC0710
SF0811
M370
M371
M372
M374
48 h


4
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RADIAL
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BC0710
SF0811
M370
M371
M372
M374
72 h

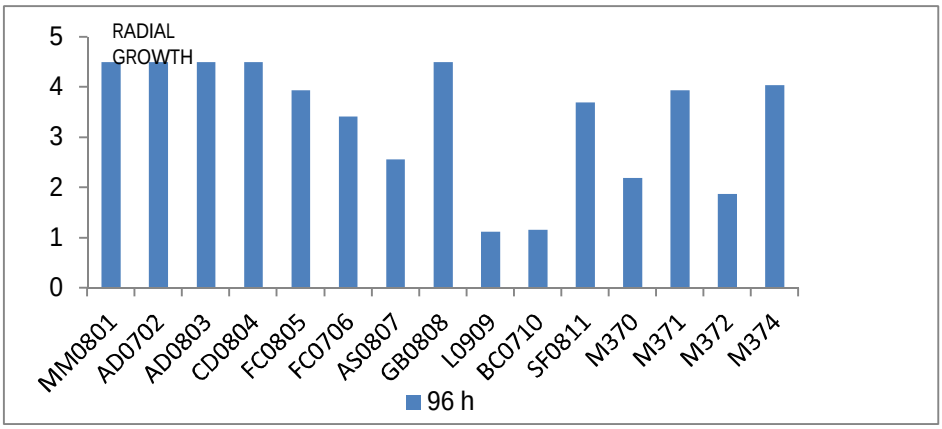

Figure 2. Radial Growth of *Metarhizium anisopliae* isolate colony at 24, 48, 72 and 96 h after medium (PDA)innoculation.

CAPÍTULO III

PATOGENICIDAD DE AISLADOS DE *Metarhizium anisopliae* SOBRE *Aeneolamia postica* EN LA REGIÓN RÍOS DEL ESTADO DE TABASCO, MÉXICO

UNIVERSIDAD JUÁREZ AUTÓNOMA DE TABASCO
"ESTUDIO EN LA DUDA, ACCIÓN EN LA FE"

2do Congreso Internacional de Agronomía Tropical y 3er Simposio Nacional Agroalimentario
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Villahermosa, Tab., a 10 de noviembre de 2011

Dictamen

Artículo en extenso --- **PATOGENICIDAD DE AISLADOS DE Metarhizium anisopliae SOBRE Aeneolamia spp EN EL ESTADO DE TABASCO, MEXICO**

Autores --- Arely Bautista Galvez, Juan F. Barrera, Emeterio Payró de la Cruz, Sergio Salgado García, Jaime Gómez Ruiz y Juan Florencio Gómez Leyva

Dictamen --- **Aceptado**

Comentarios ---. Su artículo ha sido aceptado para publicarse en el Libro Científico Tópicos Selectos en Agronomía Tropical Vol. 2.

Reciban un saludo respetuoso.

ATENTAMENTE

Dr. Juan de Dios Mendoza Palaxcios Dr. Eusebio Martínez Moreno Dr. Efraín de La Cruz Lázaro

COMITÉ CIENTÍFICO

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PATOGENICIDAD DE AISLADOS DE *Metarhizium anisopliae* SOBRE *Aeneolamia postica* EN EL ESTADO DE TABASCO, MEXICO

PATHOGENICITY OF *Metarhizium anisopliae* ISOLATES AGAINST *Aeneolamia postica* IN THE STATE OF TABASCO, MEXICO

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RESUMEN

Se evaluó la patogenicidad de 12 aislados de *Metarhizium anisopliae* (Metchnikoff) Sorokin en adultos de *Aeneolamia postica* (Walker) (Hemiptera: Cercopidae) colectados en plantaciones de caña de azúcar. La concentración probada fue de 10^8 conidias/ mL $^{-1}$. La mortalidad de los aislados de *Metarhizium anisopliae en Aeneolamia postica* fluctuó de 5 a 55% con una diferencia significativa en los aislados de 0.05 % ($F = 5.74$, $p < 0.01$). La aplicación de estos aislados como un manejo integrado de plagas es discutida en este artículo.

Palabras clave: hongos entomopatógenos, control biológico, *Aeneolamia postica.*

ABSTRACT

Was evaluated the pathogenicity of twelve isolates *Metarhizium anisopliae* (Metchnikoff) Sorokin in sugarcane froghopper *Aeneolamia postica* (Walker) (Hemiptera: Cercopidae) collected of sugarcane fields. The insects were treated with a 10^8 conidia mL^{-1} solution of each isolate. Mortality of *M. anisopliae* isolates on *Aeneolamia postica* adults ranged from 5 to 55 % There were significant differences as regards *Aeneolamia postica* mortality of isolates of 0.05 % ($F = 5.74$, $p < 0.001$). The application in fields of isolates *Metarhizium anisopliae* as an integrated control of pest is discussed in this article.

KEY WORDS: entomopathogenic fungal, biological control, *sugar cane*

INTRODUCCIÓN

La caña de azúcar dio origen a un sistema agroindustrial que ocupa un lugar preponderante y trascendente en la actividad económica y social de México, de tal manera que hoy es la agroindustria más importante (Bautista y González, 2005). Sin embargo, la producción y calidad de la caña de azúcar se ve limitada, debido a que los costos de producción se elevan y el impacto negativo al ambiente se incrementa por la aplicaciones de plaguicidas destinados al control de sus principales plagas, siendo la mosca pinta o salivazo (*Aeneolamia postica* (Walker)) (Hemiptera: Cercopidae) la más perjudicial en extensas áreas en los litorales del golfo de México y del océano Pacífico.

La mosca pinta es una de las principales plagas de la caña azúcar en la región de los Ríos del estado de Tabasco, México. En estado adulto esta plaga provoca reducciones en la producción hasta del 30%, ocurriendo la infestación de la plaga entre los meses de junio a octubre. Por lo que el productor se ve obligado a realizar hasta tres aplicaciones de insecticidas para el control. El uso de hongos entomopatógenos como *Metarhizium anisopliae* (Metchinikoff) Sorokin (Hypocreales: Clavicipitaceae) es una alternativa de control biológico (Zimmermann, 2007). Sin embargo, es importante determinar la patogenicidad de los hongos entomopatógenos en función del poder agresivo del patógeno (Robertson *et al*., 2007), para ello se requiere tener en el estado de Tabasco un aislado nativo y una concentración recomendada para el control de *Aeneolamia postica* en el cultivo de caña de azúcar. Por lo que el objetivo del presente estudio fue evaluar la patogenicidad de aislados de *Metarhizium anisopliae* del estado de Tabasco en estado adulto de *Aeneolamia postica* bajo condiciones de laboratorio.

MATERIALES Y MÉTODOS

Localización de la muestra

Se realizaron muestreos en la región de los Ríos del estado de Tabasco con la finalidad de colectar cepas de *M. anisopliae* en plantaciones de caña que abastecen al Ingenio AZSUREMEX S.A. de C.V., localizado en el km 2 de la carretera La Palma, del municipio

de Tenosique, Tabasco, México. Ubicado geográficamente entre 17^0 25´y 91^0 24´ y altitud de 60 msnm. La temperatura, precipitación y humedad relativa promedio anual es de 26^0C, 1595 mm y 83%, respectivamente. Los muestreos se realizaron entre los meses de septiembre y diciembre de los años 2007 y 2008. La toma de muestras de suelo se realizó de acuerdo con la técnica propuesta por Alameida *et al.* (1997) que consistió en tomar cinco submuestras de 0.5 kg de suelo cerca de la rizosfera del cultivo, en los primeros 10 cm de profundidad, con distribución "cinco de oro" y distantes de 5.0 cm entre las muestras. Las muestras de suelo se colocaron en bolsa de plástico y se trasladaron al laboratorio de Biotecnología de la Universidad Tecnológica del Usumacinta, en cajas de nieve seca a temperatura de 4°C. Al mismo tiempo se colectaron insectos que se encontraban micosados en las plantas de caña de azúcar en campo.

Aislamiento

Para el aislamiento de *M. anisopliae* presente en el suelo se usaron larvas de la polilla mayor de la cera (*Galleria mellonella* L.) (Lepidoptera: Pyralidae) (Vanninen, 1997). Como primer paso, las muestras de suelo se tamizaron con un cedazo de malla metálica de 2.0 mm de abertura. Enseguida se tomaron 300 g de suelo, los cuales se humedecieron al 80 % y se colocaron en un vaso de plástico de 500 mL. Después se introdujeron cinco larvas de *G. mellonella* por vaso, sellándolas con cinta adhesiva e incubándolas por siete días a 25 °C (Oberski y Tribe, 1980). Transcurrido el tiempo de incubación, las larvas se removieron y recuperaron para su observación. Las larvas muertas micosadas se recolectaron, separaron del suelo y se desinfectaron superficialmente con hipoclorito de sodio (NaClO) al 5% durante un minuto y alcohol al 70% por cinco segundos. Después se enjuagaron tres veces en agua destilada estéril en tres diferentes recipientes, removiendo el exceso de agua con papel higiénico. Por último, las larvas se colocaron en placas de petri de 100 mm x 15 mm, con una capa doble de papel filtro húmedo a las mismas condiciones de incubación antes citadas para favorecer el desarrollo de hongos (Hatting *et al.*, 1999). Cada 24 h, cada uno de los insectos micosados fue observado al microscopio estereoscópico para detectar las áreas de mayor esporulación. Los insectos micosados fueron manejados de la misma manera que se trataron las larvas de *G. mellonella* micosada. Los hongos se identificaron considerando

las estructuras reproductivas de cada uno de ellos, de acuerdo con las claves de Tulloch (1976).

Insectos

Los insectos en estado adulto se colectaron en cultivos de caña de azúcar de las 06:00 y 09:00 h con una malla entomológica, para posteriormente al laboratorio de Biotecnología de la Universidad Tecnológica del Usumacinta en frascos de 500 mL con perforaciones en la tapa superior para permitir la ventilación. Los insectos de *Aeneolamia* se alimentaron con pasto de la variedad Chontalpo durante el desarrollo del experimento.

Aislados de *Metarhizium anisopliae*

Los 12 aislados de *M. anisopliae* (Tabla 1) fueron comparados con una cepa estándar (Control), proporcionada por el laboratorio "Tiemelonlà Nich Klum" localizado en Palenque, Chiapas y un tratamiento testigo que consistió de agua destilada estéril + coadyuvante (Inex, 2 alicuotas L^{-1}). Empleando cuatro repeticiones por tratamiento. Los aislados fueron cultivados en PDA (Agar Dextrosa y Papa) por 14 d a 28^0C en completa oscuridad (Bruck *et al.*, 2005), con humedad relativa del 80%. Las conidías fueron cosechadas, la viabilidad de las esporas fue determinada para desarrollar cada bioensayo y la concentración de las esporas ajustadas (Goettel y Inglis, 1997). La patogenicidad se determinó utilizando un prototipo de caja donde se colocaron 10 insectos para cada unidad experimental. Los insectos fueron sumergidos en una concentración de 10^8 conidios/ por aislado, contenida en cajas petri durante 30 s. Los datos se registraron a las 24 h de haber iniciado el experimento. Los adultos muertos fueron removidos e incubados a 25 $\pm 1^0$C en cámara húmeda para observar el desarrollo y producción conidial del hongo y corroborar la especie inoculada. Para el análisis de los datos se usó el software Minitab Inc 2007 mediante un análisis de varianza (ANOVA) y la comparación de medias se realizó con la prueba de comparación de medias de Tukey para determinar si había diferencias entre medias de los tratamientos.

Tabla 1. Aislados de *Metarhizium anisopliae* usados en este estudio

Código de Aislamiento	Hospedante	Procedencia	Año de colecta
MM0801	*Aeneolamia postica*	Tabasco	2008
AD0702	*G. mellonella*	Tabasco	2007
AD0803	*G. mellonella*	Tabasco	2008
CD0804	*G. mellonella*	Tabasco	2008
FC0805	*G. mellonella*	Tabasco	2008
FC0706	*G. mellonella*	Tabasco	2007
AS0807	*G. mellonella*	Tabasco	2008
GB0808	*Aeneolamia postica*	Tabasco	2008
L0909[1]		TiNK	Desconocido
BC0710	*G. mellonella*	Tabasco	2007
SF0811	*G. mellonella*	Tabasco	2008
M370[2]	*Aeneolamia postica*	CNRCB	1994
M372[2]	*Aeneolamia postica*	CNRCB	Desconocido

[1] *Metarhizium anisopliae* isolate provided by the "Tiemelonlà Nich Klum" laboratory located in Palenque, Chiapas, Mexico. [2] CNRCB = Centro Nacional de Referencia en Control Biológico, Tecomán, Colima, México

RESULTADOS Y DISCUSIÓN

Se encontró que existe diferencia significativa, a las 24 h después haber aplicado el hongo a *Aeneolamia* postica ($F: 5.74 \geq 12, 39; P < 0.001$). Todos los aislados presentan patogenicidad en un rango de 5.0 a 55.0 %. En Seis de los aislados fue significativamente mayor mortalidad en *Aeneolamia postica* que el testigo, por lo que son candidatos promisorios para pasar a una segunda etapa del proceso de selección donde se realicen las pruebas de virulencia (concentración y tiempo letal cincuenta). La prueba de Tukey ($p < 0.05$) mostró que los aislados con mayor patogenicidad fueron AD0702 con un porcentaje de 55.0 %, seguido por el aislado AD0803 (52.5 %), los dos provenientes de suelo y el aislado MM0801 que presentó un porcentaje de patogenicidad del 50.0 % obtenido de *Aeneolamia postica* y el aislado CD0804 con 45.0 %, BC0710 con 42.5 % y SF0811 con

27.5 % de patogenicidad, respectivamente, obtenidos del suelo y el control con una patogenicidad del 0.0% (Figura.1).

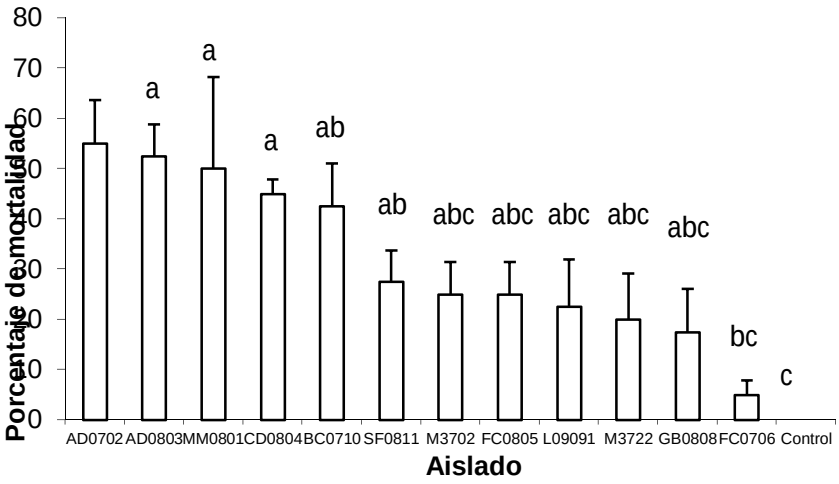

Figura. 1. Porcentaje de mortalidad en *Aeneolamia postica* con aislados de *Metarhizium anisopliae* a concentración de10^{8} conidias/ml $^{-1}$. Medias con la misma letra no son diferentes (Tukey al 5%).

El presente estudio demostró que los aislados nativos de *Metarhizium anisopliae* de la región de los Ríos del estado de Tabasco son patogénicos en *Aeneolamia postica.* Sin embargo, las aplicaciones de *M. anisopliae* podría nunca proveer el 100% del control, este debe ser como una herramienta adicional en un programa de manejo integrado (Bruck et al., 2005). La selección de estos aislamientos nativos permite contar con un método de control para *Aeneolamia.* Finalmente, para futuras investigaciones es necesario conocer la dosis letal de estos aislados para *Aeneolamia postica.*

CONCLUSIONES

En conclusión los aislados de *M. anisopliae* son patogénicos a *Aeneolamia postica* a una concentración de 10^8 conidias/mL^{-1} El uso de estos aislamientos nativos presenta una alternativa ecológica sustentable para el control de plagas en caña de azúcar. Sin embargo, es preciso continuar con estudios que permitan un mayor entendimiento en campo de la patogenicidad de estos aislados con diferentes plagas

AGRADECIMIENTO

Se agradece al Fondo-Mixto CONACYT-Gobierno del Estado de Tabasco por el financiamiento del Proyecto TAB-2007-C09-74101, el cual provienen los resultados del presente estudio. También se agradece a Javier Valle Mora por el soporte estadístico, y en especial a los productores cañeros de la región de los Ríos del estado de Tabasco por haber proporcionado información de sus cultivos.

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CAPÍTULO IV

GENETIC CHARACTERIZATION OF *Metarhizium anisopliae* (Metchnikoff) Sorokin ISOLATES FROM SUGARCANE FIELDS AND THEIR PATHOGENICITY AGAINST *Aeneolamia postica* (Walker) (Hemiptera: Cercopidae)

UNIVERSIDAD JUÁREZ AUTÓNOMA DE TABASCO
"ESTUDIO EN LA DUDA, ACCIÓN EN LA FE"

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Dictamen

Villahermosa, Tab., a 3 de septiembre de 2012

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El artículo

773UC GENETIC DIVERSITY OF *Metarhizium anisopliae* (Metchnikoff) Sorokin ISOLATES FROM SUGARCANE FIELDS AND THEIR PATHOGENICITY AGAINST *Aeneolamia postica* (Walker) (Hemiptera: Cercopidae)

ha sido aceptado para su publicación en el volumen 28 de 2012. Agradeciendo su interés por publicar en la revista, aprovecho la ocasión para enviarle un saludo respetuoso.

Reciban un saludo respetuoso.

DIR. DE INVESTIGACION POSGRADO

Dr. Efraín de La Cruz Lázaro
Editor Asociado

c.c.p. Archivo

Genetic diversity of *Metarhizium anisopliae* isolates from sugarcane fields

GENETIC CHARACTERIZATION OF *Metarhizium anisopliae* (Metchnikoff) Sorokin ISOLATES FROM SUGARCANE FIELDS AND THEIR PATHOGENICITY AGAINST *Aeneolamia postica* (Walker) (Hemiptera: Cercopidae)

CARACTERIZACION GENÉTICA DE AISLADOS DE *Metarhizium anisopliae* (Metchnikoff) Sorokin DE CAÑAVERALES Y SU PATOGENICIDAD CONTRA *Aeneolamia postica* (Walker) (Hemiptera: Cercopidae)

Fecha de recepción:
Fecha de aceptación:

ABSTRACT. A commercial strain of *Metarhizium anisopliae* (Metchnikoff) Sorokin was used by farmers in 2005 to control the sugarcane froghopper *Aeneolamia postica* (Walker) in Los Ríos, Tabasco, Mexico. The persistence and effect of this introduced isolate on the genetic diversity and pathogenicity of the native fungus population is unknown. The objective of this study was to characterise genetic diversity and pathogenicity of *M. anisopliae* isolates from sugarcane fields. The *Galleria* baiting method was used to sample fungus from the soil, whereas the insect netting method was used to capture infected *Aeneolamia postica* adults. Samples were collected from a total of 33 sugarcane fields. In total, ten isolates of *M. anisopliae* were obtained, three in 2007 and seven in 2008; seven of them were collected near to the Usumacinta River. Random amplified polymorphic DNA markers and amplified ribosomal DNA restriction analysis of fungal isolates revealed the presence of seven subgroups among the ten isolates. Two of the isolates obtained in 2008 were genetically similar to the commercial strain used in 2005. At least two genotypes of *M. anisopliae* were distributed in this region. There was no clear relationship between genotype similarity and isolate pathogenicity against *Aeneolamia postica*. Some of the native *M. anisopliae* isolates were promising candidates as agents of biological control against *Aeneolamia postica*.

Key words: *Saccharum*, entomopathogenic fungi, biological control, molecular characterization, pathogenicity

RESUMEN. En 2005, una cepa comercial de *Metarhizium anisopliae* (Metchnikoff) Sorokin fue aplicada por agricultores contra la mosca pinta *Aeneolamia postica* (Walker) en Los Ríos, Tabasco, México. Se desconoce la persistencia y el efecto de este aislado introducido sobre la diversidad genética y patogenicidad de las poblaciones nativas del hongo. El objetivo de este estudio fue caracterizar la diversidad genética y patogenicidad de aislados de *M. anisopliae* colectados en cañaverales de esta región. La técnica del insecto trampa con larvas de *Galleria* se usó para colectar el hongo del suelo, mientras que una red entomológica se usó para capturar adultos infectados de *A. postica*. Las muestras se colectaron en un total de 33 cañaverales. En total, se obtuvieron 10 aislados de *M. anisopliae*, tres en 2007 y siete en 2008; siete de los aislados se colectaron cerca del Río

Usumacinta. Marcadores del ADN polimórfico amplificado al azar y análisis de restricción de ADN ribosomal amplificado de los diez aislados, revelaron siete subgrupos. Dos de los aislados obtenidos en 2008 fueron genéticamente similares a la cepa comercial usada en 2005. Al menos dos genotipos de *M. anisopliae* se encontraron distribuidos en esta región. No hubo una clara relación entre la similitud genética y la patogenicidad de los aislados contra *A. postica*. Algunos de los aislados nativos de *M. anisopliae* son candidatos promisorios como agentes de control biológico contra *A. postica*.
Palabras clave: caña de azúcar, hongos entomopatógenos, control biológico, caracterización molecular, patogenicidad

INTRODUCTION

The production of sugarcane, *Saccharum officinarum* L., is currently the most important agroindustry in Mexico. The cultivation of sugarcane involves 812 000 ha distributed over 15 states and 57 sugar factories. With an average yield of 69 ton/ha, Mexico is one of the ten main producers of sugarcane in the world and, with a per capita consumption of 50 kg/year, it is the second highest sugar consumer. Approximately three million Mexicans depend on this economic activity, which also generates an important amount of foreign currency (Salgado et al. 2005). Approximately 28 000 ha of sugarcane are grown in La Chontalpa and Los Ríos regions in the state of Tabasco. The mean yield of sugarcane in Tabasco is 60 ton/ha (Salgado et al. 2010). The production of sugarcane is limited by the froghopper *Aeneolamia postica* (Walker) (Hemiptera: Cercopidae), the most damaging insect pest present over extensive areas of the Gulf of Mexico and Pacific Ocean coastal plains. This pest reduces sugarcane yield up to 60 % and is mainly controlled by pesticides, which increases production costs substantially (Bautista-Gálvez & Gonzalez-Cortes 2005). As the froghopper has become resistant to many insecticides, sugarcane producers in Tabasco have searched for alternative control methods. One such alternative is *Metarhizium anisopliae* (Metchnikoff) Sorokin (Moniliales: Moniliaceae), an entomopathogenic fungus that is widely used in the biological control of insect pests (Zimmermann 2007).

In 2005, some farmers applied a commercial strain of *M. anisopliae* as part of an integrated pest management strategy of *Aeneolamia postica* in the sugarcane fields of Los Ríos,

Tabasco (Bautista-Gálvez & Gonzalez-Cortes 2005). However, information on whether this strain has managed to survive in the region's sugarcane fields is lacking. Some studies indicate that *M. anisopliae* is a highly genetically diverse species (Riba et al. 1985; Fegan et al. 1993; Becerra et al. 2007; Zimmermann 2007), but in the Los Ríos region, the diversity of native populations of this fungus, their pathogenicity against *Aeneolamia postica* and their potential as biological control agents are unknown. The present study was conducted in order to improve our understanding these issues and to the genetic diversity and pathogenicity of *Metarhizium* isolates collected from the main sugarcane growing areas in the Los Rios region of Tabasco, Mexico. We used polymerase chain reaction (PCR)-based methods as the random amplified polymorphic DNA (RAPD) markers and the internal transcribed spacer (ITS-rDNA) sequence analysis for detecting genomic variability of this fungus. These methods have been applied to the study of genomic variation of *M. anisopliae* (Cobb & Clarkson 1993; Fegan et al. 1993; Bridge et al. 1997; Driver et al. 2000; Castrillo et al. 2003; Entz et al. 2005; Becerra et al. 2007; Freed et al. 2011).

MATERIALS AND METHODS

Study Area. The study was carried out in sugarcane fields found in the Los Rios region, municipality of Tenosique, Tabasco, Mexico. Sampling was performed on sugarcane fields that supply the AZSUREMEX S.A. de C.V. sugar refinery, which is located on the La Palma highway, Tenosique at 17° 25' N, 91° 24' W, and 60 m altitude, with cultivated area of 4 210 ha (Bautista-Gálvez & Gonzalez-Cortes 2005).

Fungal sampling. Samples were taken from sugarcane fields belonging to 33 farmers (two fields with and 31 without use of *M. anisopliae* to control *Aeneolamia postica*), representing 5.1 % of the total number of sugarcane producers and 4.7 % of the total sugarcane cultivated area in the Los Ríos region. On average, each farmer has 6 ha of sugarcane. This area is characterized by a Cambisol predominant soil type. Samples were taken from the soil or *Aeneolamia postica* adults. In each field, a five point sample was carried out, one in each corner of the field and another in the centre, taking the soil samples at a depth of 10 cm within the rhizosphere, as described by Almeida et al. (1997). The soil samples consisted in five 0.5 kg soil sub-samples from the upper 10 cm of soil, close to the

crop's rhizosphere with 5.0 cm distance between each sample. Soil samples were placed in plastic bags and transported to the laboratory in insulated boxes at 4 °C. Samples of *Aeneolamia postica* adults were collected from close to the base of sugarcane stems using an insect net. The captured insects were placed in Petri dishes (100 x 15 mm) and transported to the laboratory. All *Aeneolamia postica* adults were placed in a sterile Petri dish containing a moistened filter paper sealed with Parafilm to promote fungus sporulation (Goettel & Inglis 1997). The samples were collected between September and December of 2007 and 2008.

Fungal isolation. In order to isolate *M. anisopliae* from the soil, the *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae baiting method was used (Vanninen 1997). Soil samples, each of them composed by five sub-samples per field, were sieved through a 2.0 mm metallic mesh sieve and 300 g of soil was moistened and placed in a 500 mL plastic cup. Subsequently, five larvae were introduced into each cup which was sealed with sellotape and incubated for seven days at 25 °C, with a photoperiod of L: D 16 h: 8 h (Bedding & Akurst 1975; Doberski & Tribe 1980). After incubation, the larvae were examined insects were separated from the soil and disinfected in a 0.025 % sodium hypochlorite (NaOCl) solution for one min followed by 70 % alcohol for five seconds. They were then rinsed three times with sterile distilled water, and excess water removed using tissue paper (Inglis et al. 2008). The larvae were then placed in a 100 mm x 15 mm Petri dish with a double layer of damp filter paper and subjected to the aforementioned incubation conditions to favour fungus development and sporulation (Hatting et al. 1999). Mycosed insects were observed with a stereoscopic microscope to detect the body areas with highest sporulation. In order to isolate entomopathogenic fungi from field-collected samples of *Aeneolamia postica* adults, the procedures mentioned above for *G. mellonella* mycosed larvae were followed. In total, 33 soil samples and 165 *Aeneolamia postica* adults were processed. The isolated fungi were identified by using the keys elaborated by Tulloch (1976).

Fungal genomic DNA purification. Monosporic cultures were used as inoculum, that were grown on potato dextrose agar (PDA) (Lilly & Barnett 1951). Fungal inoculum was incubated and agitated for three days at 27 °C in potato dextrose broth and the mycelium was recovered on a sterile filter. The mycelium was ground with liquid nitrogen to a fine dust, which was transferred to a sterile 1.5 mL plastic microcentrifuge tube. Each sample

was mixed with lysis buffer (containing Tris HCL 50 mM, pH 7.0; EDTA 50 mM; SDS 3 %; and 1-2 β-mercaptoethanol 1 %), incubated at 65°C for 1 h, extracted with phenol: chloroform and precipitated with isopropanol (Lee & Taylor 1990). The resulting DNA was analyzed on 0.8% agarose gel in 1x SB buffer (sodium borate 10 mM), stained with ethidium bromide and visualised under ultraviolet light (UV). The DNA was cuantified by reading the optical density at 260 nm and stored at 4 °C until required (Gómez-Leyva et al. 2008).

PCR amplification of the rDNA ITS region and restriction analysis (ARDRA). The Internal Transcribed Spacer (ITS) region of the rDNA from each sample was amplified by PCR using the following reaction mixture: 1X buffer pH 8.5, ITS1 and ITS4 primers at 20 pmol each (White et al. 1990), 0.2 mM dNTPs, 2.0 mM of $MgCl_2$, 2.5 U of Taq polymerase DNA and 100 pmoles DNA with the following thermocycler conditions: 3 min at 94 ^{o}C followed by 30 cycles of: 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C with a final extension of 7 min at 72 °C. The amplified products were extracted after electrophoresis on agarose 1.2 % gel using a DNA extraction kit (Promega, Madison, WI). PCR products were ligated in the TOPO TA vector (Invitrogen) and cloned in *Escherichia coli* Top10. The recombinant TOPO vector was sequenced in both directions by the dideoxy technique and the results were compared in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

For Amplified ribosomal DNA analysis Restriction, ITS-PCR products were digested using *Hae* II (Promega. Madison, Wisconsin, USA), according to the manufacturer's instructions Restriction fragments were separated on 1.4 % agarose gel in 1x SB buffer. Ethidium bromide gel staining was performed and the DNA fragments visualized under UV light and photographed. Molecular sizes of the fragments were estimated by comparison with a 100 bp DNA ladder (Entz et al. 2005; Inglis et al. 2008).

The ARDRA technique was applied to a group of 19 fungi samples consisting of ten *M. anisopliae* isolates obtained from the sampled sugarcane fields and the following seven reference samples: one commercial strain of *M. anisopliae* from the "Tiemelonlà Nich Klum" laboratory; four *M. anisopliae* isolates (M370, M371, M372 and M374) characterized and provided by the Centro Nacional de Referencia de Control Biológico (CNRCB), Tecomán, Colima, Mexico; two *Beauveria bassiana* (Balsamo) Vuillemin

isolates and two unidentified fungi isolates obtained from the sugarcane fields sampled in this study.

RAPD-PCR analysis. Fungal isolates were characterized by RAPD analysis (Williams et al. 1990). The OPB07 (GGTGACGCAG), OPB09 (TGGGGGACTC) and OPB10 (TGCTGGGAC) primers for RAPD analysis were used (Operon Technologies Inc., Alameda, CA) (Cobb & Clarkson 1994, Guerrero et al. 2000, Kendall & Rygiewicz 2005). The reaction mixture consisted of 0.2 mM dNTPs, 10 µM of primer, 2.5 U of Taq polymerase DNA (Promega, Madison, WI) and 100 ng of DNA. Polymerase Chain Reaction (PCR) amplifications were carried out in a MJ Research thermocycler programmed as follows: 2 min at 94 °C followed by 40 cycles of 1 min at 94 °C, 1 min at 36 °C and 90 seconds at 72 °C; 7 min at 72 °C and 4°C until removed. The amplified fragments were separated by horizontal gel electrophoresis in 1.2% agarose (Ultrapure, Gibco BRL, Gaithersburg, MD). The gels were stained with ethidium bromide and photographed on a UV Cole Palmer 97500 transilluminator.

The RAPD technique was applied to a group of 10 *M. anisopliae* samples composed of the first nine isolates obtained from sugarcane fields and the L0909 commercial strain provided by the "Tiemelonlà Nich Klum" laboratory (Palenque, Chiapas, Mexico), which was used as the reference isolate.

Estimation of fungal genetic diversity. The genetic diversity of the 17 fungi samples was analyzed through polymorphism expressed by the primers. A 0, 1 matrix, referring to the absence or presence of bands in each isolate, was generated from the RAPD amplifications and ARDRA results. Variations in band intensity were not assigned as differences. The matrix was prepared using the Neighbor-Joining coefficient. Dendrograms were generated by means of a UPGMA analysis with the NTSYS 2.0 (Numeral Taxonomy and Multivariate Analysis System) program (Rohlf 1993).

Fungal pathogenicity. Netting was used to collect *Aeneolamia postica* adults in the field between 06:00 to 09:00 h. Captured insects were transported to the laboratory in 500 ml bottles with perforations in the tops to allow ventilation. They were fed with cane leaves to improve survival during the transfer. In the laboratory, insects fed on Chontalpo variety grass *Brachiaria decumbens* Stapf during the experiment. We compared the pathogenicity of nine *M. anisopliae* isolates collected in the sugarcane fields sampled in this study, two

isolates provided by the CNRCB and the standard stock provided by the "Tiemelonlà Nich Klum" laboratory. The control consisted of a sterile distilled water solution of Inex with a coadyuvant (Inex, 2 µl L^{-1}). The insects were treated with a $1X10^8$ conidia mL^{-1} solution of each isolate. Conidia concentration was determined using a hemocytometer (Goettel & Inglis 1997). Adult insects were submerged in the fungal solution contained in a Petri dish for 1 minute using a soft paintbrush. Each experimental unit consisted in a box 100 mm x 15 mm with 10 *Aeneolamia postica* insects. Four replicates were used. Insect mortality was registered 24 h after initiating the experiment. Each dead insect was placed in a 100 mm x 15 mm Petri dish with a double layer of damp filter paper and incubated to 25 ± 1 °C to favour fungus development and sporulation and to corroborate the inoculated species. The percentage of mortality was calculated by relating the number of dead insects to the total number of insects when the experiment started. The bioassays were repeated when control mortality was ≥ 20% (Alves et al. 1998). An analysis of variance and a Tukey multiple Benchmark test of averages ($P < 0.01$) was used to analyze the data. Before running the variance analysis percentage data were normalized by arcsine transformation Analyses were performed using Minitab ® Statistical Software (Minitab Inc. 2007).

Relationship between fungal genetic diversity, fungal pathogenicity and geographic distance. The Spearman's rank correlation test (Minitab Inc. 2007) was used to analyze the relationship between genetic diversity of *M. anisopliae* isolates (Jaccard coefficient), their pathogenicity against *Aeneolamia postica* (percentage of mortality) and the geographic distance between sites where the isolates were collected (Figure 1).

RESULTS

Fungal isolates collected in the sampled sugarcane fields. Fourteen fungal isolates were collected in the sugarcane field samples in this study (Table 1; Figure 1). Three of these were collected in 2007 and 11 in 2008, which represent 9.1 % and 33.3 % of sampled sites, respectively. In total, 10 isolates were morphologically identified as *M. anisopliae* (Tulloch 1976); two were identified as *B. bassiana* (Humbert 1997); two others were not identified. In 2008, two *M. anisopliae* isolates were obtained from adults of *Aeneolamia postica* collected in sugarcane fields where the reference isolate of *M. anisopliae* (L0909) was

sprayed in 2005. The other eight *M .anisopliae* isolates were obtained by the *Galleria* baiting method in sugarcane fields without *M. anisopliae* application. Most of the isolates (seven) were collected in the west of Los Ríos region, where *M. anisopliae* has never been used against *A. postica* because the pest populations are historically low.

PCR amplification of the rDNA ITS region and restriction analysis (ARDRA). Six hundred to 800 bp of DNA fragments were obtained from the PCR amplification of the rDNA ITS1-ITS4 region for the analyzed fungal isolates (Figure 2a). The size of DNA fragments of *M. anisopliae* isolates collected in sugarcane fields was very similar to those from the *M. anisopliae* reference strain L0909. All the fungal isolates gave rise to a 650 bp product, with the exception of the isolate YR0812 for which the amplicon was 800 bp in length and the isolate JC0816 with a 700 bp amplicon. The restriction rDNA of the products with *Hae* II (Figure 2b) resulted in the generation of 5 to 2 fragments in a range of 200 to 700 bp. The restriction profile of the rDNA ITS differed from those of other isolates, possibly a result of incomplete digestions. These results were not analyzed in this study.

The comparison of the obtained rDNA ITS of fungal isolates with reference to the GenBank (NCBI) database showed that isolates MM0801, AD0702, AD0803, CD0804, FC0805, FC0706, AS0807, GB0808, L0909, BC0710, SF0811, M370 and M374 were from *M. anisopliae*; and isolates AR0814 and JC0816 were from *B. bassiana*.

In the dendrogram generated from the restriction analysis (Figure 3), two neighbor clades grouped together the reference strain of *M. anisopliae* (L0909) and the ten isolates of this fungus collected in sugarcane fields. One clone were observed in each of the clades; the first clone consisted of isolates MM0801 and AD0702, and the second consisted of isolate GB0808 and the L0909 reference strain; this last clone was genetically similar to isolate AS0807. The dendrogram indicates a genetic similarity between isolates CD0804 and FC0805. Four isolates of *M. anisopliae* collected in sugarcane fields, two from each clade, formed solitary subgroups. Two of the isolates of *M. anisopliae* var. *anisopliae* provided by the CNRCB formed a separate subgroup. Both *B. bassiana* isolates (AR0814 and JC0816) were classified in the same subgroup. Finally, there were differences between the unidentified isolates YR0812 and ES0813; both also differed from the remainder of the fungal isolates.

RAPD-PCR Analysis. RAPD amplification of DNA of *M. anisopliae* isolates (Table 1) with OPB10 primer, and its dendrogram are presented in Figure 4 and 5. Genetic variation was observed amongst the isolates, with polymorphic bands ranging between 250 and 2500 bp (Figure 4). The dendrogram showed three subgroups with two isolates each and four subgroups with only one isolate each (Figure 5). The isolate FC0805 was genetically similar to the reference isolate (L0909). One of the subgroups was integrated by two isolates (CD0804 and AS0807) which were collected in two sugarcane fields that were located nearby (1.8 km), however in another subgroup the isolates (MM0801 and AD0702) were collected in two sugarcane fields separated by a distance of at least 20 km (Figure 1).

Fungal pathogenicity. Mortality of *M. anisopliae* isolates on *Aeneolamia postica* adults ranged from 5.0 % to 55.0 % (Figure 6). There were significant differences as regards *A. postica* mortality of isolates AD0702 (55.0±8.66 %), AD0803 (52.5±6.29 %), MM0801 (50.0±18.26 %), CD0804 (45.0±2.89 %), BC0710 (42.5±8.54 %) and SF0811 (27.5±6.29 %) versus control (0.0 %) ($F = 5.74$; df = 12, 39; $P < 0.001$). In this group, only the isolate MM0801 was obtained from adults of *Aeneolamia postica*; the remainder were collected using the *Galleria* baiting method.

Relationship between fungal genetic diversity, fungal pathogenicity and geographic distance. No significant association was detected between the genetic diversity of *M. anisopliae* isolates (Jaccard coefficient) and pathogenicity against *Aeneolamia postica* (percentage of mortality) ($S = 18318.84$; $P = 0.1729$). Similarly, no significant associations were detected between genetic diversity and geographic distance between sites where isolates were collected ($S = 14700.15$; $P = 0.8367$); or pathogenicity and geographic distance ($S = 17366.10$; $P = 0.3453$).

DISCUSSION

Fourteen fungi isolates were collected from sugarcane fields in the Los Rios region of Tabasco, Mexico (Table 1) distributed from east to west across the study area (Figure 1). As stated by Salgado et al. (2010), the east –west strip is wetter than the areas towards the north or south of the region. Ten of these isolates were identified as *M. anisopliae* through their morphological characteristics and by comparing their genetic sequences with the

GenBank database. The number of positive collections of *M. anisopliae* appeared to be lower in our study compared to those reported by others. For example, compared with the study by Inglis et al. (2008) conducted in British Columbia, Canada, our 10 isolates represent a lower percentage of positive cases of this fungus from the total number of analysed samples (33 % vs. 57 %). This percentage is even lower if we consider that in our study three isolates were collected in 2007 and seven in 2008. These differences are possibly explained by the environmental conditions that predominate in both studies (temperate region vs. tropical region) and also because Inglis et al. (2008) sampled a greater number of habitats than in our study, that was restricted to rural sugarcane growing areas.

Eight of the *M. anisopliae* isolates were obtained by the *Galleria* baiting method and two by collecting *A. postica* adults with a net, suggesting that this fungus was generally easier to collect from the soil, possibly because it was more abundant there. The ability of *M. anisopliae* to survive in soil is well recognized (Vanninen et al. 2000, Bidochka et al. 2001, Sallam et al. 2007, St. Leger 2008). Regarding isolate geography, both of the isolates obtained from *Aeneolamia postica* were collected to the east of the Los Rios region (Figure 1). Historically, sugarcane fields in this area suffer more damage from this pest given the close proximity to pastures, and some sugarcane farmers have used *M. anisopliae* to control *Aenolamia postica* (Bautista-Gálvez & Gonzalez-Cortes 2005). It is worth mentioning that in the west of the region, where seven *M. anisopliae* isolates were collected (Figure 1), the ground of the sugarcane fields is even wetter, as this area tends to get flooded occasionally when the Usumacinta River overflows. The higher frequency of *M. anisopliae* in this region supports reports stating that survival of the fungus is improved in wet soils (Zimmermann 2007). The presence of *B. bassiana* in the surveyed sugarcane fields was lower than *M. anisopliae*; only two isolates were obtained, one using the *Galleria* method and the other by capturing *Aeneolamia postica* adults. The lower prevalence of *B. bassiana* was possibly because this fungus is not applied to control other pests, such as the sugarcane borer *Diatraea saccharalis* (F.) in the Los Ríos region.

From the genetic point of view, the isolates were separated into two large groups or clades; one clade grouping six isolates into two subgroups of two and two subgroups of one and the other consisting of four isolates, one subgroup of two and two subgroups of one. These groupings demonstrate the existence of genetic heterogeneity between the isolates of *M.*

anisopliae collected in the study area, in agreement with the observations of other who reported high levels of genetic diversity (Riba et al. 1985; Becerra et al. 2007).

The genetic analysis suggests that two of the *M. anisopliae* isolates obtained in 2008, AS0807 isolated from *Galleria* larvae and GB0808 isolated from *Aeneolamia postica* adults, collected in the west and east of the Los Rios region respectively, and separated by a distance of approximately 20 km (Figure 1), were similar to the L0909 reference isolate, used by some producers in 2005 to control *Aeneolamia postica*. This result suggests two things; first that commercial products consisting of *M. anisopliae* can persist in sugarcane fields for at least three years after application, coinciding with experiments carried out by Milner et al. (2003) who reported a persistence of 3.5 years for *M. anisopliae* in a sugarcane crop in Australia. Second, from their origin in commercial applications, a fungus can disperse to places far away from the original treated site, possibly aided by particular farmer's practices such as the shared use of tractors to plough the soil and trucks that transport the sugarcane from the fields to the sugar refinery. Indeed, Bidochka et al. (2001) suggested that certain agricultural practices can favour the dispersion of *M. anisopliae*. Taking into consideration our results, we can infer that the L0909 isolate has spread over the Los Rios region from a few sprayings, presenting the possibility of recombination between introduced and native strains of this species, as well as the eventual impact that this phenomenon could have on the genetic diversity of *M. anisopliae* in the region and its pathogenicity towards *Aeneolamia postica*. Although the methods utilized in our study did not allow detection of recombination between fungal isolates, recombination events have been detected using isoenzyme techniques (Bidochka et al. 2001).

Genetic analysis also confirmed the existence of a native genotype of *M. anisopliae* that may be distributed across the whole region, represented by the isolates MM0801 (to the east) and AD0702 (to the west) which were collected in sugarcane fields a fair distance apart (ca. 20 km), but were genetically identical. In contrast, a genotype that was only found to the west of the region was represented by isolates AD0804 and FC0805. These findings suggest the existence of different adaptations to environmental conditions between native isolates. In this respect, Bidochka et al. (2001) pointed out that habitat can affect the genetic population of *M. anisopliae*, as in the absence of host insects the fungus possesses

the ability to survive in the soil where it is exposed to factors such as organic material, pesticides, desiccation, solar radiation and fluctuations in temperature.

The inclusion in the genetic analysis of two isolates typified as *M. anisopliae* var. *anisopliae* collected in sugarcane, one in Oaxaca, Mexico (M370) and the other in Veracruz, Mexico (M374), both provided by the CNRCB, leads us to assume that the isolates that were collected in the Los Ríos region of Tabasco do not belong to this variety of *Metarhizium* as the CNRCB isolates formed a genetically similar group but one that differed from our isolates (Figure 3).

The pathogenicity towards *Aeneolamia postica* of the *M. anisopliae* isolates collected in the Los Rios region expressed as adult mortality percentage varied from very low (5 %) to intermediate (55 %). Six of the isolates caused significantly greater *Aeneolamia postica* mortality than the control, therefore could be promising candidates for the second stage of the selection process consisting of virulence tests involving lethal concentration and mean lethal time assays; it is clear that pathogenicity studies for the selection of *M. anisopliae* isolates are a prerequisite in a biological control programme for *Aeneolamia postica*.

Contrary to other similar work that has examined the genetic diversity of *M. anisopliae*, our study explored whether pathogenicity was associated with degree of genetic similarity and geographical distance between isolates. According to our results there was no relationship between these variables. In Chile, Becerra et al. (2007) found no association between the genetic diversity of *M. anisopliae* and geographical origin. At least in the Los Rios region, there are no factors or environmental conditions that favour the spatial clustering of isolates with similar genetic and/or pathogenic characteristics; furthermore, it is not possible to forecast isolate pathogenicity in function of their degree of genetic similarity. Nevertheless, our study enabled us to identify several native *M. anisopliae* isolates that merit more detailed research due to their pathogenicity against *Aeneolamia postica* and widespread distribution across the Los Ríos region, such as the case of the MM0801 and AD0702 clonal isolates.

To determine the genetic diversity of *M. anisopliae* isolates we used RAPD markers and ITS-rDNA sequence analysis as previously studied for this fungus (Cobb & Clarkson 1993, Fegan et al. 1993, Bridge et al. 1997, Castrillo et al. 2003, Driver et al. 2000, Entz et al. 2005, Becerra et al. 2007, Freed et al. 2011). In order to study the phylogenetic

relationships within the *M. anisopliae* isolates from the Los Ríos Region, it will be needed to conduct studies that employ a multigene phylogenetic approach using near-complete sequences from nuclear encoded EF-1α, RPB1, RPB2 and β-tubulin gene regions, as it was proposed recently by Bischoff et al. (2009).

In conclusion, in the sugarcane fields of Los Ríos, Tabasco, Mexico, particularly in the wetter part of the region, a high diversity of genotypes of *M. anisopliae* exist with a wide range of pathogenicity characteristics against *Aeneolamia. postica*. It was demonstrated that the reference isolate L0909, commercially used for the control of this pest, probably persists three years after its application in the sugarcane fields of the region and can be found at sites 20 km from where it was originally applied. Finally, some of the native *M. anisopliae* isolates collected in the Los Rios region presented characteristics that make them promising candidates as agents of biological control against *Aeneolamia postica*.

ACKNOWLEDGEMENTS

We thank Fondos Mixtos CONACYT-Tabasco State Government (Project TAB-2007-C09-74101) for financial support; to Javier Valle-Mora for statistical support; and to Trevor Williams for helpful comments to the manuscript. We are especially grateful to the sugarcane producers of the Los Rios region of Tabasco for facilitating access to their sugarcane fields.

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Titles of tables

Table 1. Information on the identity, origin, and morphological characteristics of the conidia and mycelium of the studied fungi isolates.

Titles of figures

Figure 1. Map showing sampled sites and the location of the fungi isolates collected in sugarcane fields in the Los Ríos region of Tabasco, Mexico. Numbers are the isolate number in Table 1.

Figure 2. A) Products of amplification of the rDNA with primers ITS1/ITS4 in different fungal isolates from Table 1. B) Amplified products of restriction (ARDRA) with the Hae II enzyme. Lane: isolate: 1: MM0801, 2: AD20802, 3: AD30803, 4: CD0804, 5: FC0805, 6: FC0706, 7: AS0807, 8: GB0808, 9: L0909, 10: BC0710, 11: SF0811, 12: YR0812, 13: ES0813, 14: AR0814, 15: JC0816, 16: M370, 17: M371, 18: M372, 19: M374. M, 100 bp marker.

Figure 3. Dendrogram based in the amplified ribosomal DNA restriction analysis (ARDRA) in different fungal isolates from Table 1 using Jaccard coefficient.

Figure 4. Products of RAPD-PCR amplification using OPB07 and OPB09 primer. The DNA template used were (lane: isolate): 1: MM0801, 2: AD0702, 3: AD0803, 4: CD0804, 5: FC0805, 6: FC0706, 7: AS0807, 8: GB0808, 9: L0909, 10: BC0710, M: 100 bp molecular weight markers.

Figure 5. Dendrogram of fungal isolates obtained with RAPD-PCR data using Jaccard coefficient.

Figure 6. Average mortality rate (%) of adults of *Aeneolamia postica* with several isolates of *Metarhizium anisopliae* with a concentration of 1 x 10^8 conidia/ml. Means (± SE) with the same letter are not significantly different using Tukey's test at 5 %.

Títulos de tablas

Tabla 1. Información de la identidad, origen y características morfológicas de la conidia y micelio de los aislados de los hongos estudiados.

Títulos de las figuras

Figura 1. Mapa que muestra los sitios muestreados y la localización de los aislados de los hongos colectados en cañaverales de la región de los Ríos de Tabasco, México. Los números se refieren al número del aislado de la Tabla 1.

Figura 2. A) Productos de amplificación del rADN con oligosITS1/ITS4 de los diferentes aislados de hongos de la Tabla 1. B) Análisis de la digestión de los productos amplificados por PCR (ARDRA) con la enzima *Hae* II. M, marcador molecular de 100 bp. Línea: aislado: 1: MM0801, 2: AD20802, 3: AD30803, 4: CD0804, 5: FC0805, 6: FC0706, 7: AS0807, 8: GB0808, 9: L0909, 10: BC0710, 11: SF0811, 12: YR0812, 13: ES0813, 14: AR0814, 15: JC0816, 16: M370, 17: M371, 18: M372, 19: M374.

Figura 3. Dendrograma basado en la amplificación ribosomal ADN de análisis de restricción (ARDRA) en los diferentes aislados de hongos de la Tabla 1 usando el coeficiente de Jaccard.

Figura 4. Amplificación de los Productos de RAPD-PCR usando el primer OPB10. M, marcador molecular de 100 bp. El templete de ADN usado fue (línea: aislado): 1: MM0801, 2: AD0702, 3: AD0803, 4: CD0804, 5: FC0805, 6: FC0706, 7: AS0807, 8: GB0808, 9: L0909, 10: BC0710.

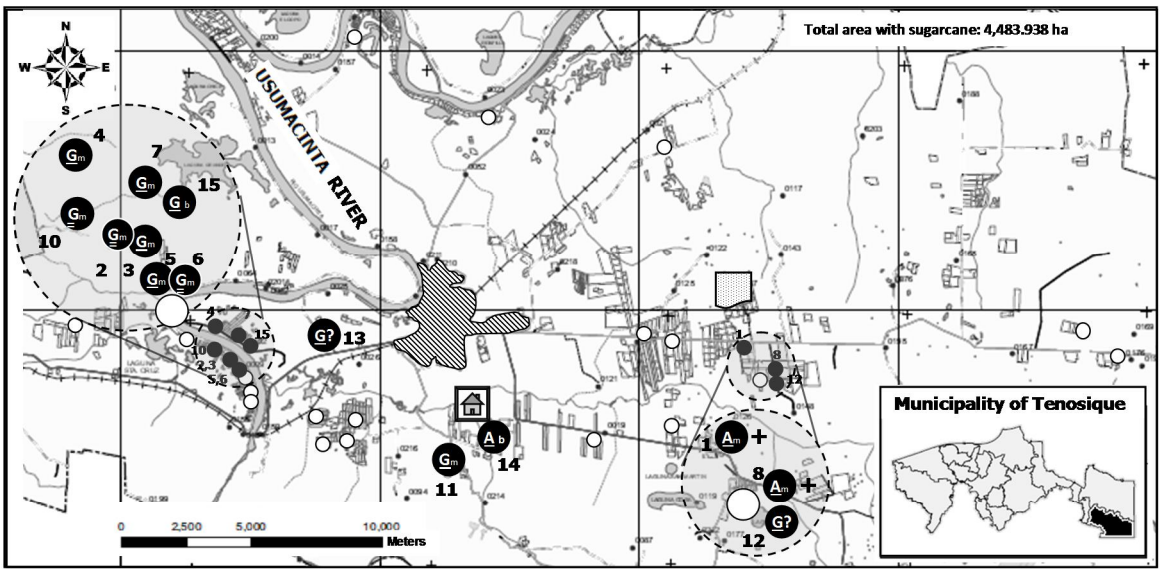
Figura 5. Dendrograma de los aislados de los hongos obtenidos con RAPD-PCR usando el coeficiente de Jaccard

Figura 6. Tasa promedio de mortalidad (%) de adultos de *Aeneolamia postica* con varios aislados de *Metarhizium anisopliae* a una concentración de 1 x 10^8 conidia/ml. Los promedios (± EE) con la misma letra no son significativamente diferentes usando la prueba de Tukey's a $p \leq 0.05$.

No.	Code	Species of insect from which the fungus was isolated	Origin of isolate	Crop from which fungus was isolated	Type of soil in sugarcane field where fungus was collected [1]	Fungus species	Year of collection	Geographical location of site where fungus was collected (UTM units)		¿The isolate L0909 was sprayed in 2005?	Length (µm) and form of conidia [2]	¿Chains of laterally adhered conidia?	Mycelium colour [3]
1	MM0801	*A. postica*	Field	Sugarcane	Fluvisol	*M. anisopliae*	2008	679170	1930877	Yes	5-8, C	Yes	DG
2	AD0702	*G. mellonella*	Field	Sugarcane	Cambisol	*M. anisopliae*	2007	659604	1930538	No	5-8, C	Yes	DG
3	AD0803	*G. mellonella*	Field	Sugarcane	Cambisol	*M. anisopliae*	2008	659604	1930538	No	5-8, C	Yes	DG
4	CD0804	*G. mellonella*	Field	Sugarcane	Cambisol	*M. anisopliae*	2008	658843	1931550	No	5-8, C	Yes	Y
5	FC0805	*G. mellonella*	Field	Sugarcane	Cambisol	*M. anisopliae*	2008	660008	1930069	No	5-8, C	Yes	DG
6	FC0706	*G. mellonella*	Field	Sugarcane	Cambisol	*M. anisopliae*	2007	660008	1930069	No	5-8, C	Yes	DG
7	AS0807	*G. mellonella*	Field	Sugarcane	Cambisol	*M. anisopliae*	2008	659689	1931128	No	5-8, C	Yes	DG
8	GB0808	*A. postica*	Field	Sugarcane	Fluvisol	*M. anisopliae*	2008	680391	1930005	Yes	5-8, C	Yes	DG
9	L0909	*A. postica*	Laboratory [4]	Unknown	Unknown	*M. anisopliae*	Unknown	Unknown	Unknown	-	5-8, C	Yes	DG
10	BC0710	*G. mellonella*	Field	Sugarcane	Cambisol	*M. anisopliae*	2007	658894	1930898	No	5-8, C	Yes	DG
11	SF0811	*G. mellonella*	Field	Sugarcane	Fluvisol	*M. anisopliae*	2008	667870	1926323	No	5-8, C	Yes	LG
12	YR0812	*G. mellonella*	Field	Sugarcane	Fluvisol	Unknown	2008	680451	1929686	No	Unknown	Unknown	W
13	ES0813	*G. mellonella*	Field	Sugarcane	Fluvisol	Unknown	2008	662991	1931168	No	Unknown	Unknown	W
14	AR0814	*A. postica*	Field	Sugarcane	Fluvisol	*B. bassiana*	2008	669459	1927224	No	1.5- 5.3 O	No	W
15	JC0816	*G. mellonella*	Field	Sugarcane	Fluvisol	*B. bassiana*	2008	659995	1930891	No	1.5- 5.3, O	No	W
16	M370	*A. postica*	CNRCB [5]	Sugarcane	Unknown	*M. anisopliae var. anisopliae*	1994	Unknown	Unknown	-	5-8, C	Yes	DG
17	M371	*A. postica*	CNRCB	Sugarcane	Unknown	*M. anisopliae var. anisopliae*	1994	Unknown	Unknown	-	5-8, C	Yes	DG
18	M372	*A. postica*	CNRCB	Sugarcane	Unknown	*M. anisopliae var. anisopliae*	Unknown	Unknown	Unknown	-	5-8, C	Yes	DG
19	M374	*A. postica*	CNRCB	Sugarcane	Unknown	*M. anisopliae*	1995	Unknown	Unknown	-	3.7-8,C	Yes	DG

[1] Salgado et al. 2010. [2] Conidia form: C = cylindrical, O = ovoid. [3] Mycelium colour: DG = dark green, LG = light green, Y = Yellow, W = White. [4] *Metarhizium anisopliae* isolate provided by the "Tiemelonlà Nich Klum" laboratory located in Palenque, Chiapas, Mexico. [5] CNRCB = Centro Nacional de Referencia en Control Biológico, Tecoman, Colima, Mexico.

Table 1
Bautista-Galvez, Barrera, Payró de la Cruz, Salgado-García, Gómez-Ruiz, Gomez-Leyva



Samples with ● and without ○ collected fungal isolates; **G**, Isolates from *Galleria* baiting method; **A**, Isolates from adultos of *A. postica*; **?**, Unidentified fungi isolates; **b**, *B. bassiana*; **m**, *M. anisopliae*; **+**, Isolate L0909 was sprayed in 2005; **A**, **G**, Isolates collected in 2007; **A**, **G**, Isolates collected in 2008; AZSUREMEX sugar refinery; Pastures; Urban zone.

Figure 1.
Bautista-Galvez, Barrera, Payró de la Cruz, Salgado-García, Gómez-Ruiz, Gomez-Leyva

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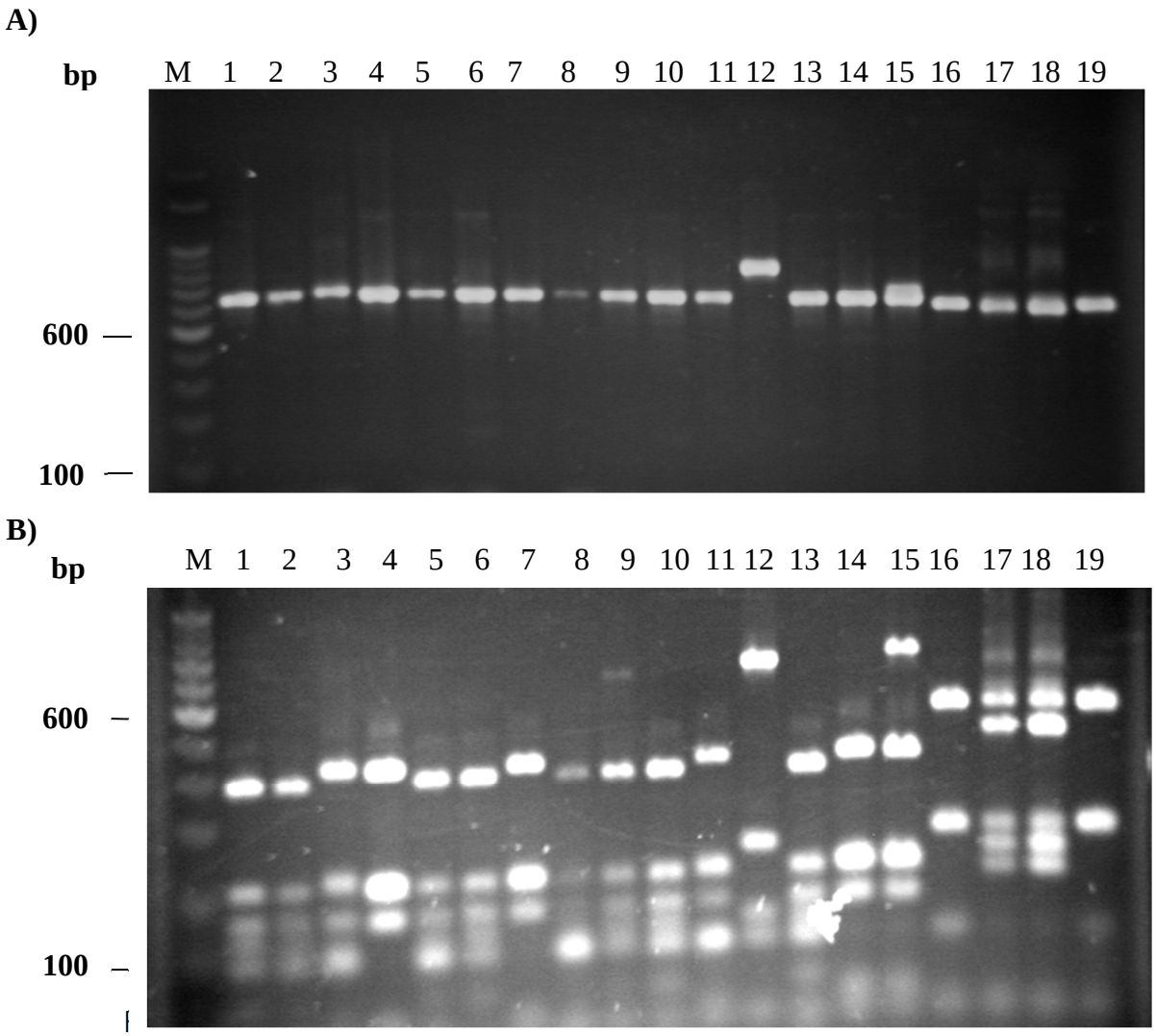

Bautista-Gálvez, Barrera, Payró de la Cruz, Salgado-García, Gómez-Ruiz, Gómez-Leyva

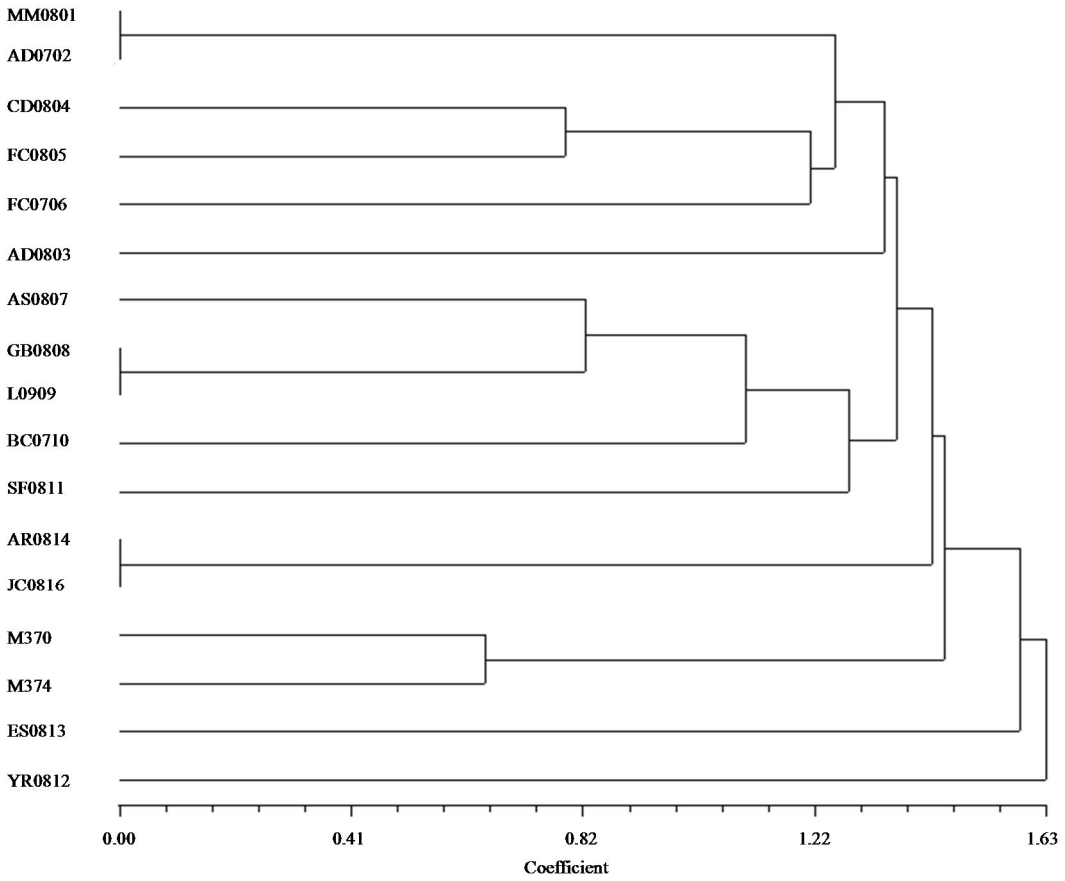

Figure 3
Bautista-Gálvez, Barrera, Payró de la Cruz, Salgado-García, Gómez-Ruiz, Gómez-Leyva

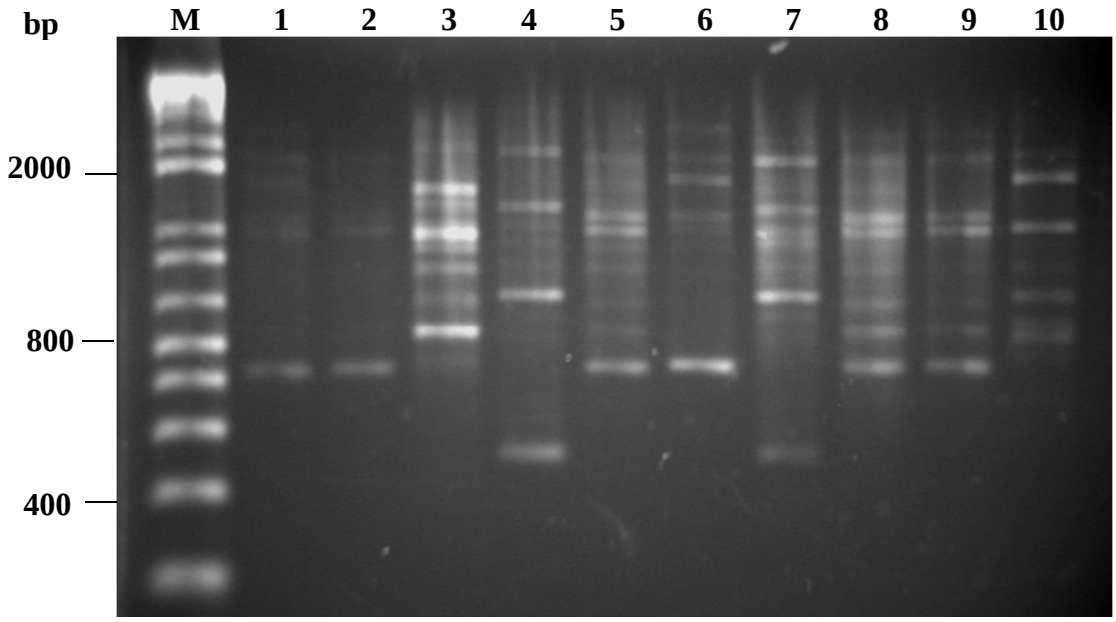

Figure 4
Bautista-Gálvez, Barrera, Payró de la Cruz, Salgado-García, Gómez-Ruiz, Gómez-Leyva

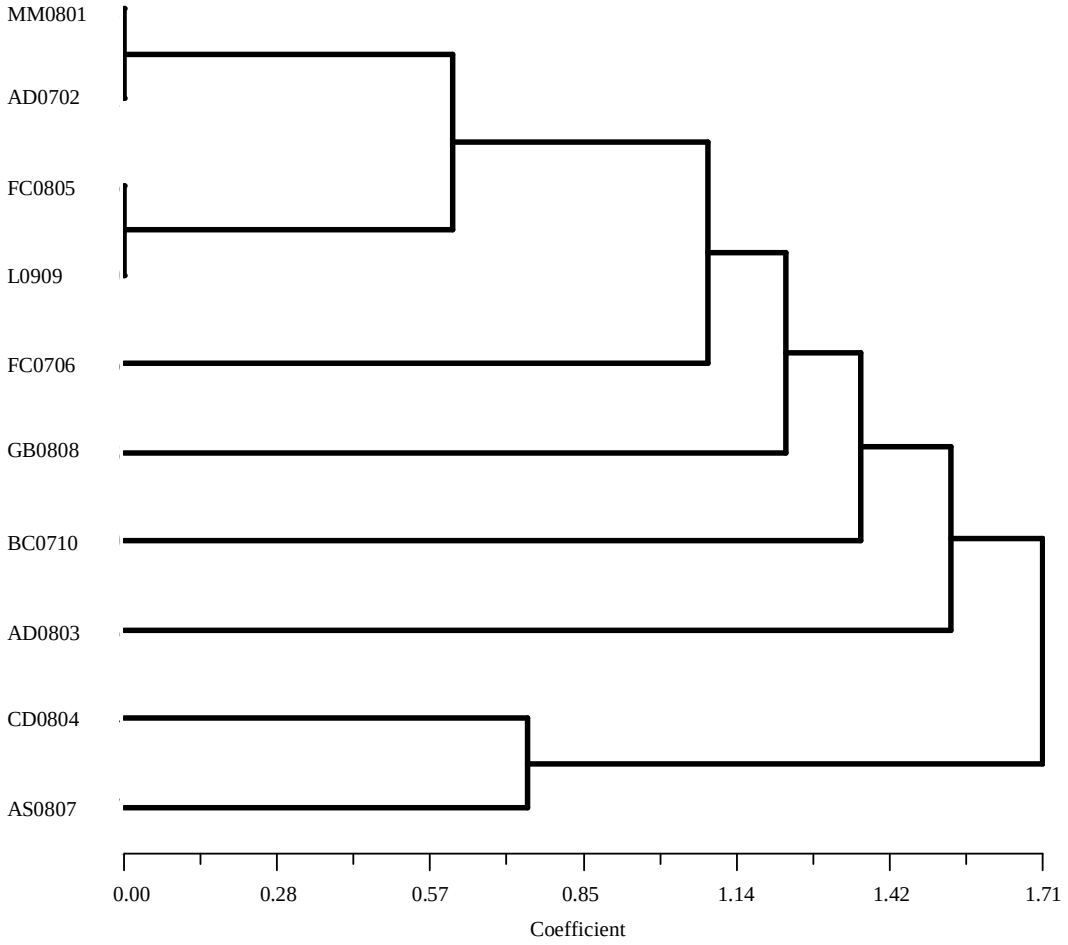

Figure 5
Bautista-Gálvez, Barrera, Payró de la Cruz, Salgado-García, Gómez-Ruiz, Gómez-Leyva

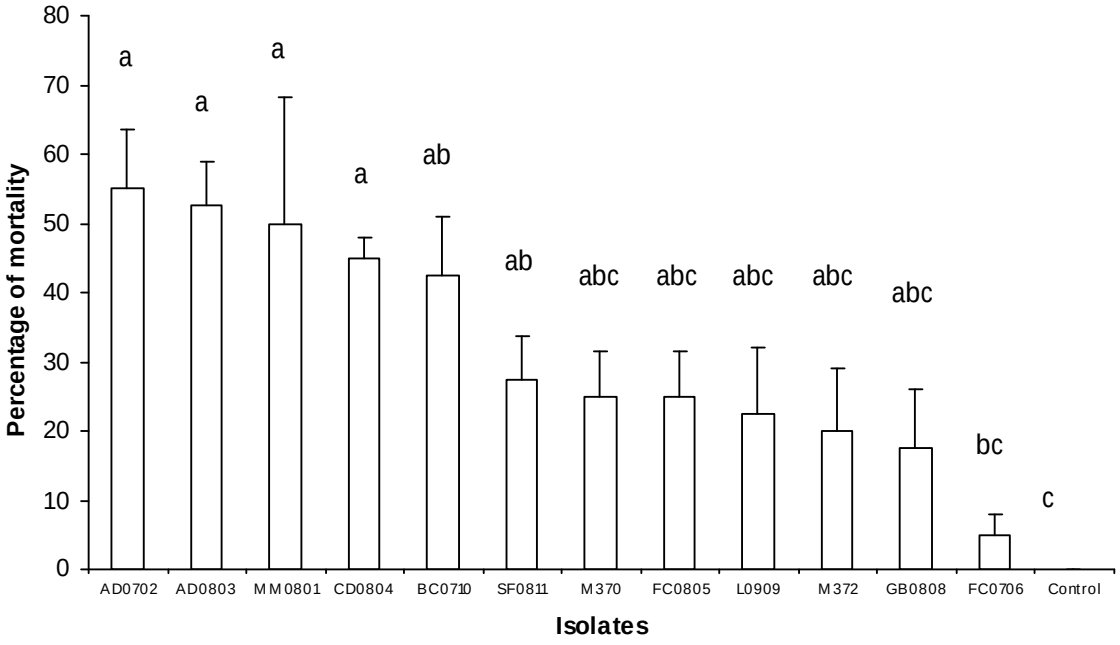

Figure 6
Bautista-Gálvez, Barrera, Payró de la Cruz, Salgado-García, Gómez-Ruiz, Gómez-Leyva

CAPÍTULO V

DISCUSIÓN GENERAL

DISCUSIÓN GENERAL

Como era de esperarse, nuestros resultados indicaron patrones genéticamente similares entre el aislado de referencia aplicado tres años atrás en cañaverales. Esto indica que el aislado 1 tiene alta probabilidad de provenir del aislado de referencia, lo cual estaría mostrando que *M. anisopliae* es capaz de persistir en el ambiente con relativamente baja variabilidad después de más de tres años de haber sido aplicado en campo. En cuanto a la persistencia, estos resultados coinciden con el reporte de Milner *et al*. (2003), quienes al estudiar diferentes formulaciones en aceite de *M. anisopliae* aplicadas en cultivos de caña de azúcar en Australia, encontraron que las conidias sobrevivieron 3.5 años después de la aplicación.

Sorpresivamente, también nuestros resultados mostraron patrones genéticamente similares entre el aislado 9 (aislado de referencia) y los aislados 5, 6, 10 y 13 procedentes de predios donde éste no se había aplicado. Esto sugiere la presencia de variedades, razas o subespecies en *M. anisopliae*, lo cual concuerda con los estudios de Fegan *et al*. (1993), quienes pudieron distinguir hasta sub especies en *M. anisopliae* var. *anisopliae*. También en este sentido, Bischoff *et al.* (2006), mencionan que *M. anisopliae* es una especie cryptica. Bidochka *et al*. (1994), al realizar un estudio de diversidad genética de *M. anisopliae* en dos regiones donde ya se había aplicado este hongo, estos autores encontraron muy baja recombinación. Según el paradigma actual, el insecto hospedero origina la influencia predominante en la genética de poblaciones de los hongos entomapatógenos (Roy *et al.,* 2010). Nuestro estudio mostró que otros factores como el tipo de suelo puede estar involucrado, ya que los aislados de *M. anisopliae* que colectamos tanto de suelo como de la mosca pinta y en los mismos lugares geográficos, presentaron baja variabilidad genética entre ellos. En este sentido, Inglis *et al*. (2008), mencionan que las ecozonas representan también un papel importante para el estudio de la diversidad genética de *M. anisopliae*. Se ha encontrado también que la presión de selección que ejercen factores bióticos y abióticos se afecta la capacidad patogénica de este entomopatógeno (Zimmermann, 2007). Estudios acerca de la variabilidad genética entre diferentes cepas de *M. anisopliae,* muestran que cepas con un mismo nivel electroforético presentaron cepas virulentas y no virulentas (Riba *et al*., 1985). De acuerdo con Hajek y

St. Leger (1994), quienes trataron de explicar la alta variabilidad genética intraespecífica observada entre aislados de *M. anisopliae*, muchas de las cepas están incluidas en unas pocas clases de genotipos geográficamente distribuidos y cuya persistencia en el tiempo y espacio sugiere que en muchas situaciones provienen de una estructura poblacional clonal.

Los resultados de este trabajo también muestran que los aislados 1, 2, 5, 6, 8, 9, 10 y 13, aunque muy similares genéticamente entre sí, no fueron idénticos; en tanto que los aislados 3, 4, 7, 11 y 12, fueron los de menor similitud genética. Este resultado muestra la ocurrencia natural de variación genética en el hongo *M. anisopliae*. Fegan *et al*. (1993), quienes realizaron un análisis RAPD en *M. anisopliae* var. *anisopliae*, el alto grado observado de diversidad genética estaría relacionado tanto con su procedencia geográfica como con los grupos de patogenicidad. Por otro lado, Hajek y St. Leger (1994), señalaron que el aislamiento geográfico y la limitada capacidad de dispersión de las esporas de *M. anisopliae*, pueden ser importantes en la evolución de los diferentes genotipos. Estos autores también mencionan que la variabilidad intraespecífica de los hongos ha sido caracterizada por diferencias en cuanto a patogenicidad, y que los casos documentados de variabilidad en la susceptibilidad del hospedero al patógeno, demuestran la ocurrencia potencial de coevolución entre el insecto hospedero y el hongo patógeno. Otros autores han descubierto que la patogenicidad de *M. anisopliae* es genéticamente distinta de acuerdo al tipo de suelo de los cañaverales (Milner, 1992) y que este hongo desarrolla diferentes adaptaciones en función al tipo de suelo y/o rizosfera donde se encuentre (Meyking y Eilenberg, 2007). Vanninen *et al.* (2000), evaluaron la persistencia de *Metarhizium anisoplae* y los autores también encontraron que existen suelos más favorables para *Metarhizium*, en nuestro estudio posiblemente podría estar influyendo el tipo de suelo, donde la mayoría de los cañaverales de la región de los Ríos del estado de Tabasco se caracterizan por tener suelos del grupo Vertisol, Lubisol y Cambisol (Salgado *et al.*,2010). Por lo que la rizosfera competente en los cultivos puede ser una característica importante para seleccionar muestras de hongos entomopatogenos (St. Leger, 2008).

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