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Physiological and molecular characterization of *Metarhizium* isolates and their acaricidal activity against *Tetranychus urticae* Koch (Trombidiformes: Tetranychidae)

Marcos Cua Basulto[1], Esaú Ruiz Sánchez[1], Horacio Ballina Gómez[1], Arturo Reyes Ramírez[1], Emanuel Hernández Núñez[3], Teresita Valencia Yah[4], Rodolfo Martín Mex[4] and Wilberth Chan Cupul[2*]

Abstract

Background: The two-spotted spider mite, *Tetranychus urticae* Koch (Trombidiformes: Tetranychidae), is one of the most damaging mites in agriculture. Due to the concern for the intensive use of synthetic acaricides, entomopathogenic fungi represents a feasible alternative to *T. urticae* management. In the present study, 7 isolates of *Metarhizium* were characterized physiological and molecularly (based on the ITS1-5.8s-ITS2 rDNA) and evaluated for their acaricidal activity [mortality, mean and 90 lethal concentration (LC_{50}: LC_{90}) and mean and 90 lethal time (LT_{50}: LT_{90})] against *T. urticae* under laboratory conditions.

Results: Sequencing of the ITS1-5.8s-ITS2 rDNA region indicated that the 7 isolates belong to *M. anisopliae*. The isolates Ma114 (3.7 ± 0.006 mm day^{-1}), Ma109 (3.5 ± 0.009 mm day^{-1}) and Ma106 (3.5 ± 0.006 mm day^{-1}) had the highest radial growth rate and Ma114 ($92.2 \pm 0.86\%$) and Ma108 ($94.4 \pm 1.07\%$) had the highest germination percentage. All isolates were pathogenic to *T. urticae*, causing mortality that ranged from 45.3 to 85.3%. The LC_{50} and LC_{90} were 1.2 and 2.8, 1.1 and 2.5, and 1.2 and 2.8×10^8 conidia mL^{-1} for isolates Ma110, Ma109 and Ma106, respectively, while the LT_{50} and LT_{90} were 7.7 and 16.5, and 7.2 and 16.1 days for isolates M110 and Ma109, respectively.

Conclusion: The isolates Ma110 and Ma109 of *M. anisopliae* were moderately pathogenic and virulent against *T. urticae*.

Keywords: *Tetranychus urticae*, Entomopathogenic fungus, *Metarhizium*, Microbial control, Bioassays

Background

The two-spotted spider mite (*Tetranychus urticae* Koch) is one of the most damaging pests in agriculture. Damage by *T. urticae* is typical in leaves, but at high population density inflorescences and fruits are also affected. Infested plants undergo a decrease in photosynthetic rate, plant growth and fruit production (Landeros et al. 2013).

To control *T. urticae*, synthetic acaricides have been intensively used, but even though this strategy has alleviated the problem to some extent, issues associated with the continuous use of these chemicals include a decrease in population of beneficial arthropods, increased risk for human health and selection of acaricide resistant populations (Shin et al. 2017).

The use of biological control agents such as entomopathogenic fungi (EPF) represents an alternative to reduce the dependence of synthetic acaricides to control of phytophagous mites. Among EPF, *Metarhizium* spp. (Ascomycota: Clavicipitaceae) are considered

*Correspondence: wchan@ucol.mx
[2] Facultad de Ciencias Biológicas y Agropecuarias, Universidad de Colima, Km 40 autopista Colima-Manzanillo, 28934 Tecomán, Colima, Mexico
Full list of author information is available at the end of the article

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an effective biological control agent against a wide range of phytophagous mites (Souza et al. 2014). In laboratory assays, *Metarhizium anisopliae* has shown high activity against *T. urticae* with mortalities range from 80 to 100%, when mites were exposed to 1.0×10^7 to 1.0×10^8 conidia mL^{-1} (Dogan et al. 2017). In the field and greenhouse conditions, *M. anisopliae* at concentration of 1×10^8 conidia mL^{-1} had also showed efficacy to reduce the population of *T. urticae* (Bugeme et al. 2014a). Also, *Metarhizium* has been proven to cause mortality on insects from different orders including: Hemiptera, Heteroptera, Coleoptera, Lepidoptera, Thysanoptera, Orthoptera, Diptera and Isoptera (Carolino et al. 2014). In addition, it has already used in commercial formulation of bioinsecticides for pest insects of foliage, root and stored grain (Kepler and Rehner 2013). Due to the importance of the biological control of two-spotted spider mite, the objective of this study was to characterize physiological and molecularly fungal isolates of *Metarhizium* and evaluate their acaricidal activity against the *T. urticae*.

Methods

Fungal isolates

Seven isolates of *Metarhizium* were obtained from the Laboratory of Biological Control of the Faculty of Biological and Agricultural Sciences at the University of Colima in Mexico. The isolates were obtained from soil samples in the states of Colima and Jalisco, Mexico (Table 1). The fungi were grown on Sabouraud dextrose agar (SDA, MCD® Lab, Mexico) in Petri dishes under laboratory conditions 25 ± 1 °C. To obtain the spore suspension, the conidia were harvested from the surface of a fungal colony (12 days old) by scrapping using 10 mL of sterile distilled water plus Tween 80 (0.05%), and then, it was filtered with a sterile gauze to avoid the mycelium and to recover only the conidia (Chan-Cupul et al. 2010). The conidia suspension was used for the bioassays.

Table 1 Source and origin of the Metarhizium isolates

Isolates	Origin (Rizosphere soil)	Locality and state
Ma114	Open field	"Loma alta," Alcaraces, Colima
Ma110	Open field	"Cerro Grande," Quesaría, Colima
Ma109	Open field	"El Tanque," Quesaría, Colima
Ma106	Sugarcane	"Escobera," Trapiche, Colima
Ma103	Sugarcane	"Pastores," Colima
Ma97	Sugarcane	"Rio Colorados," Pihuamo, Jalisco
Ma91	Open field	"El atracadero" Colima, Colima

Molecular identification of *Metarhizium* spp.

Genomic DNA extraction was performed from monosporic cultures in GPY culture medium, according to the method developed in the GeMBio laboratory (Tapia-Tussell et al. 2006). The ITS1-5.8s-ITS2 region of the rDNA was amplified using the primers ITS1 (5′ TCC GTAGGTGAACCTGCGG 3′) and ITS4 (5′TCCTCC GCTTATTGATATGC 3′) (White et al. 1990), at a final volume of 50 µL, which they contained 25 ng of genomic DNA, 0.20 mM of each dNTP (Invitrogen), 1.5 mM of $MgCl_2$, 1 µM of primers and 1 U of *Taq* DNA polymerase (Invitrogen). DNA amplification was performed in a GeneAmp 9700 DNA thermal cycler (Perkin-Elmer). PCR conditions were: 94 °C for 1 min; 30 cycles at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min; and 5 min at 72 °C (Tapia-Tussell et al. 2008). The PCR products were sequenced at Macrogen Inc. (Seoul, Korea). The sequences were compared with the database of the gene bank of the *National Center for Biotechnology Information* (s. f.) in the *Basic Local Alignment Search Tool* (BLAST) program and a phylogenetic tree was elaborated with the MEGA software version 10 with the neighbor-joining (Kumar et al. 2018).

Physiological characterization

The radial growth and conidial germination were used as parameters of physiological characterization of the *Metarhizium* isolates (Permandi et al. 2020). To evaluate the radial growth rate (RGR), 5 µL of a conidial suspension of 1×10^6 conidia mL^{-1} of each isolate was inoculated individually in the center of a Petri dish with SDA. The conidia suspension was obtained from an 11-day-old colony in a Petri dish, 10 mL of sterile distilled water with Tween (0.05%) was deposited on the surface of the colony and scraped with a sterile spatula, and conidia were recovered by filtration in a falcon tube (50 mL) with sterile gauze. The conidia concentration was counted and adjusted in a Neubauer chamber. Petri dishes were incubated at 25 ± 1 °C and photoperiod of 16: 8 h light: darkness. The diameter of the growing colony was measured daily for 10 days using a Vernier, with the colony diameter values were calculate the radial growth rate (mm d^{-1}) (Chan-Cupul et al. 2010). A Petri dish served as a replicate and 10 replicates per fungal isolate were used.

The evaluation of conidial germination of each isolate was evaluated using the same Petri dishes from the RGR test. Ten microliters of 1×10^6 conidia mL^{-1} were deposited on SDA Petri dishes. The plates were incubated at 25 °C. Conidia germination was recorded each 12 h by 3 times (36 h) in a microscope (40×), 100 conidia for each plate were counted. Germinated conidia were considered when the germ tube has the same length than the conidia

(Ayala-Zermeño et al. 2015). Ten replicates (Petri dishes) were used per fungal isolates. All bioassays were repeated twice.

Acaricidal activity of fungal isolates

A stock culture of *T. urticae* was established on common bean plants (*Phaseolus vulgaris* L.) into an entomological cage under greenhouse conditions (minimum temperature) = 24.0 °C, maximum temp = 38.5 °C, average temp = 31.4 °C, minimum relative humidity (RH) = 43.15%, maximum RH = 73.97%, average RH = 58.5% and 12:12 h of light: darkness photoperiod). For bioassays, fungal spore suspensions were obtained by 15-day-old colonies grown in SDA as previously described by Ayala-Zermeño et al. (2015). To test the pathogenicity of the fungal isolates, an initial assessment was conducted using a spore concentration of 1×10^7 conidia mL^{-1}. Then, for the most active fungal isolates, a range of spore concentrations (1×10^7, 1×10^6, 1×10^5 and 1×10^4 conidia mL^{-1}) was evaluated as described by Jeyarani et al. (2011). Spore suspensions were sprayed until runoff onto both surfaces of *P. vulgaris* leaves using hand sprayer. The leaves were air-dried for 20 min under the laminar flow cabinet, and then placed on wet cotton wool in Petri dishes. Adult mites (20 individuals per Petri dish) were then placed onto the treated leaves. Control leaves were sprayed with sterile distilled water containing 0.05% Tween 80. Petri dishes were placed in an incubator at 25 ± 1 °C, 75 ± 5% RH, and photoperiod of 16:8 h light: darkness. Mortalities were recorded daily for 12 days. Dead mites were transferred and kept for 15 days in Petri dishes lined with moist filter paper to observe mycosis (Eken and Hayat 2009). Each Petri dish served as a replicate, 8 replicates per fungal isolate were used.

Data analysis

All experiments were set in a completed randomized design. Data subjected to analyses of variance were checked for normality and homoscedasticity. Significant differences ($P < 0.05$) among means were determined by the Tukey test. Mean and ninety lethal times (LT_{50} and LT_{90}) and mean and 90 lethal concentrations (LC_{50} and LC_{90}) were calculated using Probit analysis. All analyses were performed in the Statistical Package for Statgraphics®.

Results

Molecular identification

Sequencing of the ITS1-5.8s-ITS2 rDNA gene showed that the 7 isolates had homology with *M. anisopliae* (Metschn.) Sorokin 1883, 6 isolates with identity of 99.8–100% and the MA91 isolate of 99.2%, identity were obtained from BLAST algorithm NCBI database (Zhang et al. 2000). Phylogenetic analysis showed that isolates MA97, MA106, MA114 and MA109 were very close to each other, while isolates MA103 and MA110 shared the same clade, and isolate MA91 shared a clade with another isolated of *M. anisopliae* (Fig. 1).

Physiological characterization of fungal isolates

The radial growth ($F = 83.01$; $df = 6$; $P < 0.001$) and the germination of conidia ($F = 120.69$; $df = 6$; $P < 0.001$) varied significantly within fungal isolates. The highest radial growth rates were observed in the isolates Ma114 (3.7 mm day^{-1}), Ma109 (3.5 mm day^{-1}) and Ma106 (3.5 mm day^{-1}). The germination of conidia was significantly higher in the isolate Ma114 (92%) relative to the other isolates, their values ranged from 62.6 to 84.6% (Table 2).

Acaricidal activity of fungal isolates

The mortality of *T. urticae* adults caused by *Metarhizium* isolates (1×10^7 conidia mL^{-1}) varied significantly ($F = 16.84$; $df = 7$; $P < 0.001$). The highest mortalities of *T. urticae* were caused by the isolates Ma110 (83.5 ± 4.1%), Ma106 (80.8 ± 5.3%) and Ma109 (74.6 ± 7. 8%). In contrast, the isolates Ma103 (52.5%), Ma97 (48.3%) and Ma91 (56.4%) achieved the lowest mortalities (Fig. 2).

The mean lethal time (LT_{50}) at the concentration of 1×10^7 conidia mL^{-1} for all *Metarhizium* isolates was calculated (Table 3). The lowest values of LT_{50} were achieved by the isolates Ma110 (7.7 days) and Ma109 (7.2 days); in contrast, the highest value of LT_{50} was achieved by the isolate Ma97 (13.2 days). The values of LT_{50} for the remaining isolates ranged from 9.7 to 11.7 days (Table 3). Regarding to the LT_{90}, both the *M. anisopliae* isolates Ma110 and Ma109 achieved the lowest values with 16.5 and 16.1 days.

The isolates Ma110, Ma109 and Ma106 were evaluated in a dose–response experiment (1×10^4 to 1×10^7 conidia mL^{-1}). Nonsignificant differences were observed among isolates for both, the LC_{50} and the LC_{90}. The calculated LC_{50} for Ma110, Ma109 and Ma106 were 1.2, 1.1 and 1.2×10^8 conidia mL^{-1}, respectively, and the calculated LC_{90} were 2.8, 2.5 and 2.8×10^8 conidia mL^{-1}, for Ma110, Ma109 and Ma106, respectively (Table 4).

Discussion

In order to find a more safety method to control the two-spotted spider mite, this study evaluated the acaricidal activity of *Metarhizium* isolates against *T. urticae*. In addition, *Metarhizium* isolates were molecularly physiologically characterized. Phylogenetic analysis showed variability among strains, isolates Ma103 and Ma110 were grouped in the same clade, while isolate Ma91 was more distant and shared the clade with *M. anisopliae*

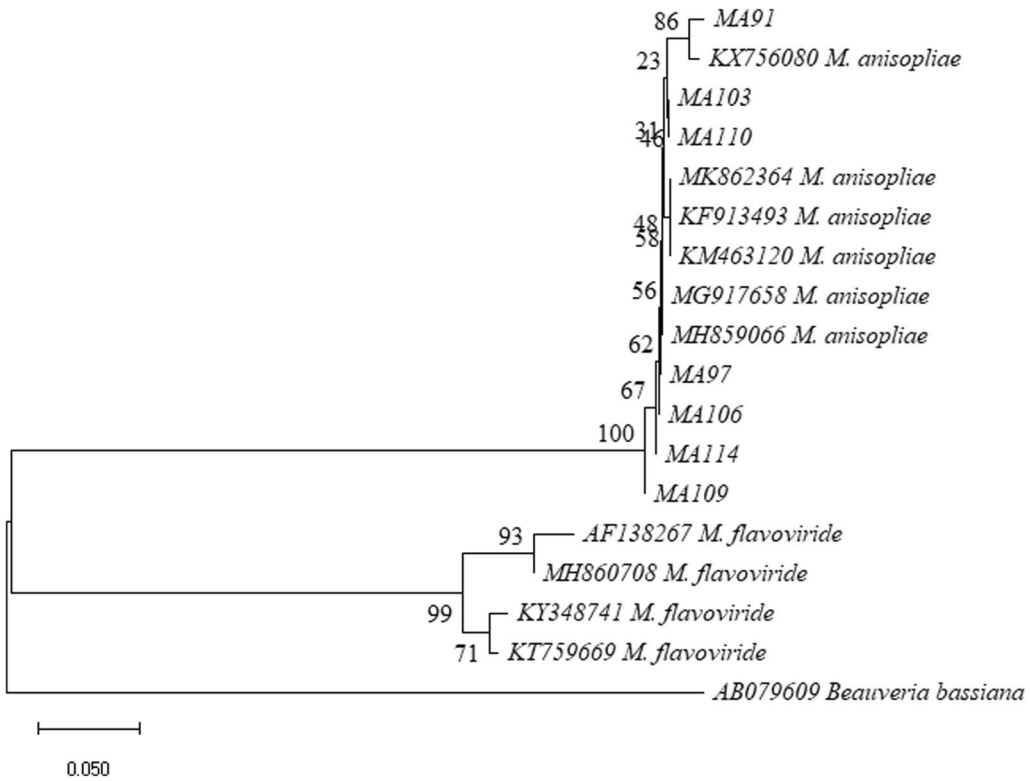

Fig. 1 Phylogenetic relationship of seven isolates of *Metarhizium* spp. The tree was constructed from the analysis of ITS-5.8s rDNA gene sequencing by the neighbor-joining method

Table 2 Radial growth rate and conidial germination (mean ± standard error) of seven *Metarhizium* spp. isolates

Isolates	Radial growth rate (mm day^{-1})	Conidial germination (%)
Ma114	3.7 ± 0.006a	92.2 ± 0.86a
Ma110	2.2 ± 0.005c	75.4 ± 0.81c
Ma109	3.5 ± 0.009a	83.6 ± 0.92b
Ma106	3.5 ± 0.006a	83.2 ± 0.86b
Ma103	1.9 ± 0.0014d	84.6 ± 0.92b
Ma97	2.9 ± 0.005b	74.0 ± 1.00c
Ma91	2.1 ± 0.004 cd	62.6 ± 0.67d

Different letters indicate significant differences between all *Metarhizium* strains. ANOVA and Tukey test ($p \leq 0.05$)

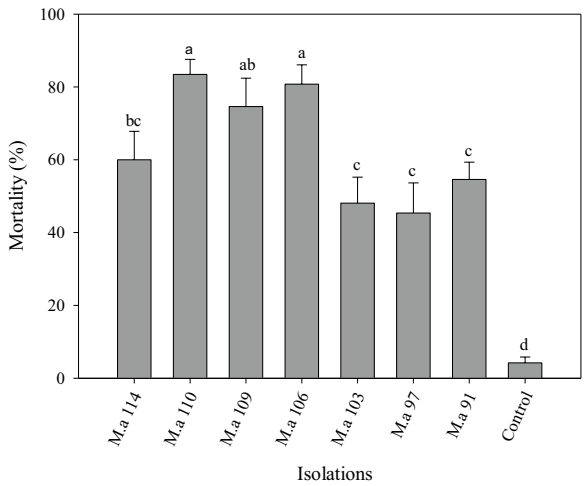

Fig. 2 Mean (± standard error) mortality of *Tetranychus urticae* adults exposed to 1×10^7 conidia mL^{-1} of seven isolates of *Metarhizium anisopliae* under laboratory conditions. Bars with different letters are significantly different (Tukey test, $n = 5$, $p < 0.05$)

KX756080. The distance among the isolates may suggest a new variety, as documented by Pantou et al. (2003) when amplified the ITS1-5.8S-ITS2 rDNA region of *Metarhizium* isolates. It must be taken into consideration that *Metarhizium* is a genus with a wide genetic diversity, because it is a cosmopolitan microorganism and adapts to different ecosystems and niches. In addition, it has different survival strategies, being a saprobic, entomopathogenic and endophyte genus (Ramírez-Milanes et al. 2022).

Table 3 Mean (LT_{50}) and 90 (LT_{90}) lethal time of *Metarhizium anisopliae* isolates on *Tetranychus urticae* treated at a concentration of 1×10^7 conidia mL^{-1} in the laboratory

Isolates	LT_{50} (days) (95% fiducial limits)	LT_{90} (days) (95% fiducial limits)	Probit equation	X^2	*P* value
Ma114	9.7 (8.1–13.0)ab	22.2 (17.3–32.1)ab	$Y = -0.9970 + 0.1027x$	31.83	0.0000
Ma110	7.7 (6.8–9.1)a	16.5 (14.0–21.0)a	$Y = -1.1063 + 0.1441x$	64.79	0.0000
Ma109	7.2 (6.5–8.5)a	16.1 (13.6–20.1)a	$Y = -1.0490 + 0.1447x$	64.55	0.0000
Ma106	10.0 (8.0–14.9)ab	26.0 (19.1–42.6)ab	$Y = -0.8097 + 0.0809x$	20.94	0.0000
Ma103	9.9 (8.0–14.0)ab	24.1 (18.3–37.2)ab	$Y = -0.8935 + 0.0902x$	25.28	0.0000
Ma97	13.2 (10.2–20.9)b	28.4 (20.7–48.3)b	$Y = -1.1128 + 0.0842x$	19.14	0.0000
Ma91	11.7 (8.9–19.8)ab	29.8 (21.1–56.1)b	$Y = -0.8226 + 0.0704x$	15.52	0.0001

Different letters indicate significant differences according to the overlap of the fiducial limits

Table 4 Mean (LC_{50}) and 90 (LT_{90}) lethal concentration of *Metarhizium anisopliae* isolates against *Tetranychus urticae* treated at a concentration of 1×10^7, 1×10^6, 1×10^5 and 1×10^4 conidia mL^{-1} in the laboratory

Isolates	LC_{50} (conidia mL^{-1}) (95% fiducial limits)	LC_{90} (conidia mL^{-1}) (95% fiducial limits)	Regression equation	X^2	*P* value
Ma110	1.2×10^8 ($1.1 \times 10^8 - 1.4 \times 10^8$)a	2.8×10^8 ($2.5 \times 10^8 - 3.1 \times 10^8$)a	$Y = -1.0049 + 8.1619x$	338.971	0.0000
Ma109	1.1×10^8 ($9.9 \times 10^7 - 1.2 \times 10^8$)a	2.5×10^8 ($2.4 \times 10^8 - 2.7 \times 10^8$)a	$Y = -1.0081 + 9.2559x$	437.337	0.0000
Ma106	1.2×10^8 ($1.1 \times 10^8 - 1.4 \times 10^8$)a	2.8×10^8 ($2.5 \times 10^8 - 3.1 \times 10^8$)a	$Y = -1.0126 + 8.1865x$	339.409	0.0000

Different letters indicate significant differences according to the overlap of the fiducial limits

The physiological characterization of *Metarhizium* isolates indicated significant differences among isolates in radial growth and conidial germination. This result could be due by the genetic diversity of *Metarhizium* or at their biochemical activity, because fungal growth can be stimulated by the culture media (Gandarilla-Pacheco et al. 2012). In this regard, the present results are similar to those reported by Dimni et al. (2004) in *M. anisopliae* isolates, which achieved a mycelial growth from 2.3 to 3.2 mm day^{-1}. In contrast, Nussenbaum et al. (2013) reported higher values for radial growth rate for *Metarhizium* isolates from 4.0 to 5.3 mm day^{-1}.

Fungal growth rate is an important characteristic to select isolates for biological control programs, because it is expected that isolates with the highest growth rate would also have high acaricidal effects. Talaei-Hassanloui et al. (2007) suggested that there was a positive association between fungal radial growth and fungal virulence in EPF. Another important predictor of the acaricidal effect of a fungus is the conidial germination rate. In the present study, this variable ranged from 62.6 to 94.4% at 24 h post-inoculation. In another studies, Bugeme et al. (2009) observed in *M. anisopliae* 86–96% of conidial germination at 24 post-inoculation. Likewise, Onsongo et al. (2019) found similar results, and observed that the highest rate of conidial germination was achieved at temperatures of 25–30 °C. The rate of conidial germination is considered an important indicator of fungal virulence, based on the assumption that a spore on the insect cuticle that germinates rapidly would also have higher probability to penetrate and initiate the infection process in the host insect (Andersen et al. 2006); in *M. anisopliae* a positive relationship between fungal virulence and rate of conidial germination has been well documented (Ummidi et al. 2013). However, this asseveration is not a general rule, because in the present study *M. anisopliae* Ma114 achieved the highest RGR and conidia germination, but the isolate was not the most virulent.

All fungal isolates evaluated in this work caused significant mortality against *T. urticae* adults. Similar to other studies, the mortality caused by the fungal isolates ranged from 45.4 to 83.5% using 1×10^7 conidia mL^{-1}. For example, Bugeme et al. (2014b) found 65 to 100% mortality using a spore concentration of 1×10^7 conidia mL^{-1}. However, Chandler et al. (2005) observed that *M. anisopliae* isolates caused no more than 43% of mortality of *T. urticae* using 1×10^7 conidia mL^{-1}.

In the present study, the calculated LT_{50} ranged from 7.2 to 13.2 days using a conidial suspension of 1×10^7 conidia mL^{-1}. The lowest values for LT_{50} were observed for the isolates Ma110 (7.7 days) and Ma 109 (7.2 days). These values were relatively high compared to those reported in other studies, where calculated LT_{50} for *Metarhizium* spp. was within 2.2–4.0 days (Castro et al. 2018). Regarding to the LT_{90}, Bugeme et al. (2009) reported values from 3.1 (ICIPE48) to 11.7 (ICIPE97)

days for *M. anisopliae* isolates from Kenya. In other study, Hassan et al. (2017) reported 8.7 and 20.0 days for LT_{90} in *M. anisopliae* isolates from Egypt. In the tomato spider mite (*Tetranychus evansi* Baker & Pritchard), the LT_{90} values for *M. anisopliae* isolates ranged from 7.3 to 15.1 days (Vitalis et al. 2005), these values are less than those observed in the present study. These variations in LT_{50} and LT_{90} could be due to the genetic diversity of *M. anisopliae*; this genus is cosmopolitan with a versatile lifestyle as saprobe, endophyte, entomopathogen and antagonist of fungal plant pathogens. Recently, Serna-Domínguez et al. (2019) deported that Colima State has a wide genetic diversity of *M. anisopliae* including isolates with wide geographical distributions and different lifestyle.

By other hand, the calculated LC_{50} for the most pathogenic isolates (Ma110, Ma109 and Ma106) ranged from 1.1×10^8 to 1.2×10^8 conidia mL^{-1}. These values are similar to those reported by Hassan et al. (2017), who documented LC_{50} of 9.3×10^7 and 4.57×10^8 conidia mL^{-1} for *M. anisopliae* isolates on *T. urticae*. In contrast, the LC_{50} values in the present study were relatively higher than the LC_{50} values of 2.0×10^5 to 5.0×10^5 conidia mL^{-1} reported in previous studies by Elhakim et al. (2020). Regarding the LC_{90}, the isolates Ma110, Ma109 and Ma106 values that ranged from 2.5 to 2.8×10^8 conidia mL^{-1}) were lower than those (LC_{90}, 2.24 to 2.85×10^{10} conidia mL^{-1}) reported by Elhakim et al. (2020). Taken both, the LT_{50} and LC_{50} as indicator of the fungal virulence, obtained fungal isolates may be considered moderately virulent. It is important to note that the virulence of EPF not only depends on intrinsic characteristics of the fungal isolates, but also on the concentration and frequency of the applications, as well as on the environmental conditions (Oyku et al. 2017).

Conclusions

Metarhizium anisopliae isolate Ma114 achieved the highest germination percentage and the isolates Ma114, Ma109 and Ma106 reached the highest mycelial growth rate. According to the sequencing of the ITS1-5.8s-ITS2 region (rDNA), the all studied isolates were identified as *Metarhizium anisopliae* (Metschn.) Sorokin 1883. The most pathogenic isolates were the *M. anisopliae* Ma110 and Ma106 that caused 83.46 and 80.76% mortality of *T. urticae*. According to the LT_{50}, the most virulent *M. anisopliae* isolates against *T. urticae* were the Ma110 (7.7 days) and Ma109 (7.2 days). The calculated LC_{50} for the isolates Ma110 and Ma109 were 1.2 and 1.1×10^8 conidia mL^{-1}, with nonsignificant difference between them.

Abbreviations

EPF: Entomopathogenic fungi; ITS: Internal transcribed spacer; rDNA: Ribosomal deoxyribonucleic acid; LC_{50}: Mean lethal concentration; LC_{90}: Ninety lethal concentration; LT_{50}: Mean lethal time; LT_{90}: Ninety lethal time; SDA: Sabouraud dextrose agar; Ma: *Metarhizium anisopliae*; PCR: Polymerase chain reaction; BLAST: Basic local alignment search tool; RGR: Radial growth rate; ANOVA: Analyses of variance; X^2: Chi-square test.

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Authors' contributions

All authors contributed equally in the manuscript. MCB developed the bioassays and wrote the manuscript. ERS and WCC isolated and identified morphologically the fungal strains. TVY and RMM identified molecularly the fungal strains; ARR and HBG analyzed the collected data and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data are available in the manuscript, and the materials used in this work are of high transparency and grade.

Declarations

Ethics approval and consent to participate

Not applicable. This manuscript is in accordance with the guide for authors available on the journal's website. Also, this work has not been published previously and is approved by all authors and host authorities. This article does not contain any studies with human participants or animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Tecnológico Nacional de México, Antigua Carretera Mérida-Motul Km. 16.3, Yucatán 97345 Conkal, Mexico. [2]Facultad de Ciencias Biológicas y Agropecuarias, Universidad de Colima, Km 40 autopista Colima-Manzanillo, 28934 Tecomán, Colima, Mexico. [3]Departamento de Recursos del Mar, CINVESTAV-IPN Unidad Mérida, 97310 Mérida, Yucatán, Mexico. [4]Laboratorio GeMBio, Centro de Investigación Científica de Yucatán A.C., 97200 Mérida, Yucatán, Mexico.

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