High susceptibility of *Tetranychus merganser* (Acari: Tetranychidae), an emergent pest of the tropical crop *Carica papaya*, towards *Metarhizium anisopliae s.l.* and *Beauveria bassiana* strains

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Abstract

Background.
Mites are one of the main causes of phytosanitary problems for *Carica papaya* crops. *Tetranychus merganser* is considered to be an emerging pest of various crops in tropical countries. It is one of the most detrimental pests in the papaya orchards of some regions of Mexico. The current field control of *Tetranychus* spp. involves the extensive use of chemicals that have some degree of toxicity to humans or the environment and may cause selection of resistance. The use of biological alternatives such as parasitoids and mite predators have limited effectiveness. In order to find effective but non-toxic alternatives for mite pest management, bio-products that are able to be mass produced and applied to large production areas have been sought, including the entomopathogen fungi. *B. bassiana* and *M. anisopliae* s.l. are the fungi most extensively used for the biological control of insect pests. Although they do not cause natural epizootic diseases in mites, there are reports that show that they infect *T. urticae*, and should be evaluated for use in the biological control of papaya's mite pests.
Methods. The family, genus, and species of the mites collected in papaya orchards were identified by taxonomic keys. A *T. merganser* colony was established and the susceptibility of adult females to thirty entomopathogenic fungi strains was evaluated under laboratory conditions with an *in vitro* mass screening bioassay on papaya leaf discs. Ten strains of *Metarhizium anisopliae* sensu lato (s.l.), eleven of *Beauveria bassiana*, nine of *Lecanicillium* sp. and one of *Hirsutella thompsonii* var. *sinematosa* were tested. The infectivity of adult females was evaluated calculating the percentage of mortality. To calculate the LC_{50} and LT_{50} of the most virulent strains, a bioassay was performed using serial concentrations (1×10^4 - 1×10^8

39   conidia/mL) for each strain. Strains showing ability to infect eggs laid were evaluated with a
40   novel egg-infectivity bioassay. The internal transcribed spacer (ITS) region of the more lethal
41   strains were sequenced.

42   **Results**. *T. merganser* and *T. urticae* were found in orchards of *Carica papaya* (Maradol variety
43   and Tainung hybrid) in Campeche, Mexico. All tested strains of *M. anisopliae* s.l. and *B.*
44   *bassiana* were infectious to the adult female of *T. merganser* at a concentration of 1×10^8
45   conidia/mL. Six strains belonging to the PARB clade of *M. anisopliae* (Ma002, Ma003, Ma004,
46   Ma005, Ma014 and Ma034) caused 100% mortality, and one strain of *B. bassiana* (Bb016)
47   caused 95% mortality. The most virulent was Ma034, with an LC_{50} of 1.73×10^6 conidia/mL
48   followed by Ma005 and Ma003. Ma005 and Ma034 were the fastest strains to reach $LT_{50,}$
49   achieving this in less than 3.7 days. Additionally, Ma034 and Ma014 strains were infectious to
50   more than 70% of the eggs.

51   **Conclusions**. *T. merganser* and *T. urticae* are present in the papaya orchards of Campeche,
52   Mexico. The high susceptibility of *T. merganser* adult females and eggs toward several *M.*
53   *anisopliae* s.l. or *B. bassiana* strains suggests that these fungi are a viable alternative to control
54   this emergent pest. The most virulent strain, Ma034, was also infective to eggs, and is the most
55   promising to be tested in the field.

56

Introduction

58   Papayas are produced in more than 60 tropical countries, with India, Brazil, Indonesia, Nigeria,
59   Mexico, Ethiopia, and Guatemala being the main producers (FAO, 2020). Mexico is the third
60   leading producer of papaya, representing 7.3% of the world's production (SIAP, 2020); it is also
61   the main exporter. The United States and Canada are its highest importers of this crop
62   (SAGARPA, 2020).

63   The mite complex is currently considered to be one of the most important phytosanitary
64   problems for papaya crops in Mexico (Pantoja et al., 2002). *Tetranychus* spp. is a complex of red
65   spider mites, which includes the cosmopolite *T. urticae*. This is the most important pest for many
66   crops due to the losses that it causes and its resistance to chemical pesticides (Van Leeuwen et
67   al., 2010; Adesanya et al., 2021). In the Yucatan Peninsula, a papaya producing and exporting
68   region of Mexico, the presence of the red mite has been reported by producers as an increasingly
69   problematic pest. This problem is being addressed with pesticides that have different modes of
70   action, such as growth inhibitors (clonfetezine and etoxazole), nerve transmission inhibitors
71   (amitraz, spirodiclofen, avermectin), lipid sites (spiromesifen, spirotetramat), mitochondrial
72   activity (acequinocyl, bifenazate, fenpyroximate), chitin synthesis (buprofezin), as well as
73   ovicides and spiracle blockers such as oil paraffine and propylene glycol monolaurate. However,
74   according to the IRAC website (https://irac-online.org/pests/tetranychus-urticae/), the resistance
75   to avermectin, acequinocyl, bifenizate and fenpyroximate has already been reported.
76   Additionally, some of these acaricides are toxic and producers apply them without knowledge
77   about exactly which specie or species of mites are present. The correct identification of mites in

78   the complex affecting a papaya crop field is necessary to select the most appropriate pest

79   management strategies (Abato-Zárate et al., 2014).

80   *Tetranychus merganser* Boudreaux is a species that was not considered to be a significant pest

81   because its distribution was restricted to the United States and Mexico and its presence was

82   occasionally reported on plants of low economic importance such as *Ligustrum vulgare*,

83   *Solanum nigrum*, and *Solanum rostratum* (Tuttle et al., 1976). However, since the last decade of

84   the 20th century, its presence has been registered in China (Wang and Ma, 1993), Thailand (Ullah

85   and Gotoh, 2014), and it has been related to phytosanitary problems crops including chili peppers

86   *Capsicum annuum* (Estébanes-Gonzalez & Rodríguez-Navarro, 1991), peanut *Arachis hypogaea*

87   (Rodríguez, 1999) or cactus *Opuntia ficus-indica*, in Mexico (Lomeli-Flores et al., 2008;

88   Rodríguez-Navarro, 1999). It has gained recent attention in the production zones of *Carica*

89   *papaya* L., and it has been found in papaya orchards of several distant regions along the Gulf of

90   Mexico (Figure 1). It may be the most injurious mite pest for papaya in some regions (Valencia-

91   Domínguez et al., 2011; Reyes-Perez et al., 2013; Abato-Zárate et al., 2014; López-Bautista et

92   al., 2016). Thus, *T. merganser* is considered to be an emerging pest which is expanding

93   geographically and in terms of its host range (Lomelí-Flores et al., 2008; Valencia-Domínguez et

94   al., 2011; Monjarás-Barrera et al., 2015, 2017).

95   Tetranychid mites are characterized by high fertility, arrhenotokous parthenogenesis, and short

96   life cycles at high temperatures. Their oviposition in the abaxial side of leaves can vary with the

97   temperature or feeding (Dehghan et al., 2009). At temperatures higher than 23 ºC, *T. merganser*

98   can reduce the time of all its developmental stages (egg, larva, nymph and adult). Therefore, in

99   the tropics it is present in all seasons but its eggs lose viability at temperatures greater than 35 ºC

100   (Hoy, 2011; Reyes-Pérez et al., 2013). Its shorter life cycles, in combination with the improper

101   handling of pesticides and their excessive use, encourages the selection of resistant populations

102   and the reduced effectiveness of chemical acaricides (Kim et al., 1998; Mota-Sanchez and Wise,

103   2022).

104   An alternative to the chemical control of pests is the use of biological agents such as

105   entomopathogenic fungi. These biological agents are important contributors to the natural control

106   of insect populations and have proven efficient in controlling pests in the field (Siongers and

107   Coosemans, 2003; Avery et al., 2008). In Mexico, several products of microbial origin are used

108   in a complementary manner alongside chemical pesticides to prevent population growth at levels

109   above damaging thresholds (Barrera et al., 2008; Cortez-Mondaca, 2008; Hernández-Velázquez

110   and Toriello, 2008).

111   Entomopathogenic fungi were named as such, considering that most of them are insect

112   pathogens. However, since the beginning of the last century, fungi naturally infecting Acari have

113   been reported worldwide (Mietkiewski et al., 2000), such as species of the *Neozygites* and

114   *Hirsutella* genera, which infect members of the Tarsonemidae, Eriophyidae, and Tetranychidae

115   families (Yaninek et al., 1996; Chandler et al., 2000; Van Der Geest, 2000; Lopes et al., 2009).

116   Although these genera are specific for mites, they have characteristics that limit their mass

117   production, commercial sale and use in the field (Hajek, 1997; Poinar and Poinar, 1998). For

118   example, some species are obligate parasites and have low conidial formation, limiting the
119   inoculum production; and their hyphal bodies are fragile; these characteristics make it difficult to
120   formulate. Additionally these species requires a long establishment period when they are used on
121   pre-infected mites to inoculate a field (Chandler et al., 2000; Hountondji et al., 2010).
122   *Beauveria bassiana* and *Metarhizium anisopliae* s.l. are the most commonly used fungi for pest
123   control worldwide because they are facultative and pathogenic to a wide range of insects. Thus,
124   it is feasible to isolate and cultivate them in the laboratory, mass-produce them on different
125   substrates and easily apply them in the field at high spore densities. Although *B. bassiana* and *M.*
126   *anisopliae* s.l. have not been found to infect mites naturally (Wekesa et al., 2015), and they do
127   not cause natural epizootic diseases in mites, some isolates of *B. bassiana* and *M. anisopliae*
128   have been shown to be infective to *T. urticae* under laboratory conditions, when inoculated in
129   greenhouses, or in the field. This is suggestive of their potential to control this pest (Alves et al.,
130   2002; Chandler et al., 2005; Draganova and Simova, 2010; Bugeme et al., 2014; Bugeme et al.,
131   2015; Dash et al., 2018; Yesilayer, 2018). Despite the growing number of articles showing that
132   *B. bassiana* or *M. anisopliae* s.l. are capable of infecting *Tetranychus* under controlled
133   conditions, there are no reports to-date regarding the susceptibility of *T. merganser* to these
134   fungi. The identification of highly-effective strains with which to infect a specific plague is a
135   requirement for its application in biological control.
136   In this work, we confirm the presence of both *T. urticae* and *T. merganser* in papaya plantations
137   of Campeche State, in the Yucatan Peninsula, Mexico. We report a high susceptibility of *T.*
138   *merganser* to several strains of *M. anisopliae* s.l. and one *B. bassiana* also isolated in Campeche.
139   The adult female mortality and egg infection data reported here suggest the strong potential of
140   these entomopathogenic fungi in the biological control of this emerging pest in the field.
141

Materials & Methods

142
143   **Sampling and identification of mites in papaya orchards of Campeche, Mexico**
144   During 2017 and 2018, five papaya orchards located in the state of Campeche, Mexico were
145   monitored for the presence of mites (Table 1). We sampled one hectare in each orchard that was
146   previously identified by the producers as a site with a possible mite presence. Fifteen plants were
147   randomly selected within that area and two mature leaves were sampled from the lower stratum
148   of each plant. The leaves were transported to the laboratory in a plastic bag inside a container.
149   All mites present on the abaxial surface of the sampled leaves were observed with a stereoscope
150   and collected to be identified. The specimens were incubated in lactic acid as a clearing agent,
151   for seven days. Subsequently, the males were mounted on microscope slides with Hoyer´s
152   mounting medium (Henderson, 2001). The taxonomic keys of Gerson et al. (2003) were used to
153   identify the families of the collected mites. The Tuttle et al. (1976) keys were used for
154   identification at the genus and species level (Fig. 1A).
155   **Establishing a *T. merganser* colony**
156   *T. merganser* were collected in papaya fields and were raised on thirty 15-day-old plants of
157   *Phaseolus vulgaris* (Fabaceae) var. Jamapa. The plants were watered every third day. The mite

158   colony was protected from external depredators by containing them inside cages built with a 200

159   μm mesh. The colony was raised at 27-28 °C, 67-77 % relative humidity (RH), and a 12:12 h

160   (light:dark) photoperiod. The density of the mite colony was maintained at approximately 200

161   mites per leaf. Plants severely damaged by mite infestation were replaced with new plants.

162   **Entomopathogenic fungal strains**

163   Thirty strains belonging to the entomopathogenic fungi collection of the Microbial Pest Control

164   Laboratory of the Postgraduates College (Colegio de Postgraduados, Campus Campeche) were

165   tested against *T. merganser*. A total of 11 strains were identified as *B. bassiana* (Bb005, and

166   Bb014 to Bb023) and ten as *M. anisopliae* sensu lato (Ma002 to Ma009, Ma014, and Ma034)

167   using conidiophores morphological keys. The *M. anisopliae* s.l. and *B. bassiana* strains were

168   isolated from the soil or infected insects that were collected from several agricultural fields or

169   semi-conserved rain forest areas from Campeche. Nine strains of *Lecanicillium* sp. isolated from

170   *Onychiurus folsomi* (Collembola: Onychiuridae) collected in Mexico City (19°18' 58.59" N; 99°

171   11' 30.12"W) were also tested, as well as the CHE-CNRCB 377 strain of *Hirsutella thompsonii*

172   var. *sinematosa* from the collection of the National Reference Center for Biological Control

173   (Centro Nacional de Referencia de Control Biológico) (SENASICA, 2016) isolated from

174   *Phyllocoptruta oleivora* (Acari: Eriophyidae).

175   The internal transcribed spacer (ITS) region of seven strains identified in this work as the more

176   lethal for *T. merganser* (Ma002, Ma003, Ma004, Ma005, Ma014, Ma034, Bb016) were

177   amplified by PCR using 100 ng of gDNA (Raeder and Broda, 1985), 40 pmol of ITS1 and ITS4

178   primers (White et al, 1990), and 30 μL of high fidelity supermix (cat 10790-020; Invitrogen,

179   Waltham, MA). The PCR cycles consisted of 1 min at 95 °C, 55 °C and 72 °C with 2 min at 95

180   °C for initial denaturation and 7 min at 72 °C for the final extension. The PCR products were

181   purified with the QIAquick PCR Purification Kit (cat. 28104; Qiagen, Hilden, Germany) and

182   sequenced by an external service provider. The ITS sequences were compared by BLASTn

183   analysis with NCBI (nr/nt) and UNITE 8.3 (Nilsson et al., 2019) databases. The *M. anisopliae*

184   s.l. ITS sequences were aligned with ITS sequences of reference strains from UNITE/NCBI

185   databases with Clustal Omega (1.2.4) and a guide tree was constructed. A phylogenetic tree was

186   constructed with MEGA X using the UPGMA and Neighbor-Joining method with 1,000

187   replicates.

188   The strains of *Beauveria*, *Metarhizium*, and *Lecanicillium* were grown in potato dextrose agar

189   and incubated at 28°C until conidiogenesis occurred. *H. thompsonii* was grown in Sabouraud

190   dextrose agar with yeast extract and transferred to medium H (Cabrera et al., 2006) for

191   sporulation. The conidia were recovered from sporulated cultures with 0.01% triton in sterile

192   distilled water by scraping the surface. The suspension was homogenized and the conidia were

193   counted in a Neubauer chamber to adjust the concentration. The percentage of viable conidia was

194   also determined. Isolates with more than 90% germination were used in bioassays.

195   **Screening of fungal pathogenic strains on adult *T. merganser* females**

196   To evaluate whether any *B. bassiana*, *M. anisopliae* s.l., or *Lecanicillium* sp. strains from our

197   collection or the *H. thompsonii* strain from SENASICA were pathogenic to *T. merganser*, we

198   performed a mass screening bioassay evaluating mortality on adult females (Fig. 1B). For this
199   infectivity bioassay, papaya leaf disks of 3 cm in diameter were disinfected under aseptic
200   conditions in a solution of sterile distilled water with sodium hypochlorite (0.06%) and 10 μL of
201   liquid soap, and continually stirred for 1 min. Leaf disks were rinsed three times with sterile
202   distilled water and subsequently allowed to dry on a sterile paper towel. Each leaf disk was
203   immersed in 2 mL of 1×10^8 conidia/mL of each strain for 30 s, before allowing it to dry on a
204   sterile paper towel. This conidia concentration was previously selected by testing three strains at
205   1×10^4, 10^6, and 10^8 conidia/mL. We observed a mortality of more than 50% only at the highest
206   concentration. All strains of the *Lecanicillium* sp. showed very low sporulation levels, thus we
207   used 1×10^7 conidia/mL for bioassays. The viability of the conidia of all tested strains was
208   evaluated before each experiment. The inoculated disks were placed upside down on an Oasis®
209   floral sponge base of $3.6 \times 3.3 \times 0.5$ cm inside a 5 cm diameter Petri dish containing 3 mL of
210   water. Twenty adult *T. merganser* females were placed onto each disk using a fine brush. In the
211   control group the disks were immersed in sterile distilled water with 0.01% triton. The Petri
212   dishes were kept closed and maintained at 28 °C with 50-55% RH and a 12:12 h (light:dark)
213   photoperiod. On the third day, the mites were transferred onto a new leaf disk without inoculum.
214   The mortality of the mites was quantified daily from the third to the sixth day post-infection. The
215   mortality caused by mycosis was verified by the microscopic observation of specific fungal
216   sporulation on the mummified mites (Fig. 1C). Three independent assays comparing all strains
217   were performed. The average percentage and the standard deviation of adult mortality caused by
218   each strain were calculated and then plotted with Sigma Plot 14.0. Afterwards, the percentage of
219   mortality was arcsine-transformed to normalize the data before one-way analysis of variance
220   (ANOVA) was performed. Differences between the means were compared by Tukey tests, and
221   p=0.05 was considered as significant. To estimate the lethal time to 50% mortality (LT_{50}) with
222   the screening assay data, the accumulated mortality recorded at the concentration of
223    $1x10^8$ conidia/mL for each strain were corrected for natural mortality (Abbott, 1925) and used to
224   estimate LT_{50} using the Probit function (Finney, 1971) with a significance level of 0.05%. The
225   statistical analyses of all bioassays in methods section were carried out using Minitab 14
226   Software (Pennsylvania State College, PA: Minitab, Inc., 2003).
227   **LC_{50} & LT_{50} of the most virulent strains**
228   The seven more virulent strains (causing 90-100% mortality of *T. merganser* in less time) were
229   selected to perform a bioassay similar to the one described above. Each strain was evaluated in
230   serial concentrations from $1x10^4$ to $1x10^8$ conidia/mL, from the third to the seventh day. In this
231   range, the recorded mortality was between 0 and 100%. For each strain, at least six independent
232   experiments were performed and the lethal concentration to 50% mortality (LC_{50}) was estimated
233   and the LT_{50} was recalculated using the Probit analysis with a significance level of 0.05%.
234   **Infectivity on eggs**
235   The strains that showed the ability to infect eggs laid by the females during the screening assay
236   (Fig. 1d) were evaluated with a novel infectivity bioassay. Sixty adult females were placed on a
237   disinfected papaya leaf fragment (8 x 5 cm) for 24 h to lay eggs. The females were removed by

238   washing the leaves with 0.05% triton with strong agitation for 1 min. The leaves were allowed to
239   dry in a laminar flow cabinet. Twenty eggs were carefully recovered with a fine brush and placed
240   onto 1 cm^2 of sterilized screen-printing mesh (130 μm). The mesh was previously placed onto 1
241   cm^2 of a sterilized cotton pad and both were placed onto a sterilized glass slide. The eggs were
242   separated with the brush, while avoiding contact between the eggs (Fig. 1E). A volume of 1.5 μL
243   of a 1×10^8 conidia/mL suspension was added onto the glass slide, which was sufficient to cover
244   all eggs, allowing the excess liquid to be absorbed by the cotton pad. Sterile distilled water with
245   0.01% triton was added to eggs in the control group. Each slide was placed onto a cotton pad
246   moistened with sterile distilled water to promote fungal growth, inside a 15 mm Petri dish. The
247   Petri dishes were kept at 28 °C, 50-55% RH, and a 12:12 h (light:dark) photoperiod. Infectivity
248   was recorded on the fifth day based on the presence of mycelia on the eggs (Fig. 1F) and by
249   verifying the characteristics of the spores of each fungus. The fungi were stained with
250   lactophenol cotton blue at 0.5%. Three independent experiments were performed. The average
251   percentage and the standard deviation of infectivity on eggs were calculated, and plotted (Sigma
252   Plot 14.0). The percentage of infectivity was arcsine-transformed and a one-way analysis of
253   variance (ANOVA) was performed. Differences between the means were compared by Tukey
254   tests; p=0.05 was considered as significant.
255

256 ## Results

257 **Identification of *T. merganser* and *T. urticae* in papaya orchards of Campeche, Mexico**
258   Five papaya orchards located in two distant sites in the Campeche State were sampled repeatedly
259   over two years, reaching a total of twelve samples (Figure 1). The presence of two *Tetranychus*
260   species was recorded: *T. merganser* and *T. urticae*. On some sampling dates, we did not find any
261   mites, possibly because the producers use acaricides to control the pest. *T merganser* was found
262   in high density in three of the five sampled orchards growing on two different papaya varieties:
263   Maradol and a Tainung hybrid. *T. urticea* was observed in less density than *T. merganser*, when
264   it was present. The characteristics confirming the identification of species from the *Tetranychus*
265   genus are the distance between the pair of duplex setae, the presence or absence of tenant hair,
266   and the morphology of the aedeagus knob. The aedeagus knob of *T. urticae* has an anterior
267   projection acute and a rounded shape in posterior projection, their females are greenish (Fig. 2b,
268   2d). The aedeagus of *T. merganser* resembles the head and neck of a goose (Fig. 2c); the
269   pretarsus do not have tenant hair, and each empodium ends in a tuft of three pairs of hair. The
270   specimens that were identified as *T. merganser* had the unique coloration of its species, namely
271   bright red females and pale yellowish males (Fig. 2a).
272   Given that *T. merganser* has been previously reported in *C. papaya* orchards of Yucatan and
273   Veracruz (Fig. 1), other states located along the Gulf of Mexico (Valencia et al., 2011; Abato-
274   Zárate et al., 2014; Monjarraz-Barrera el al., 2017), we looked for biological control for this
275   emerging pest to avoid the use of chemical acaricides and resistance selection.
276

277 **Screening of fungal pathogenic strains on adult *T. merganser* females**
278   The *H. thompsonii* strain that was tested showed no infectivity in *T. merganser* adult females at
279   1×10^8 conidia/mL, even on the sixth day post-inoculation. However, many strains in our

280 collection were infectious towards adult *T. merganser* females, indicating a considerably
281 different mortality effect between species (Fig. 3). The *M. anisopliae* s.l. strains caused the
282 highest *T. merganser* mortality levels in comparison to the other fungal species (Fig. 3A). All
283 these strains resulted in significant differences in mortality, compared to the control (F=74.85, p
284 < 0.0001) (*Data S1*). Five *M. anisopliae* s.l. strains (Ma002, Ma004, Ma005, Ma014 and Ma034)
285 caused mortality to 100% of mites, Ma003 to 91%, and the other strains (Ma006, Ma007, Ma008
286 and Ma009) caused mortality from 75% to 85%.
287 In the case of *B. bassiana* (Fig. 3b), all strains were infectious to adult females, but only seven of
288 the eleven strains showed mortality with statistically significant differences compared to the
289 control (F=17.55, p < 0.0001) (*Data S1*). Three strains (Bb005, Bb014, Bb016) showed the
290 highest mortality levels, reaching 95% in the case of Bb016. The Bb015, Bb019 and Bb021
291 strains showed a mortality of only 40-43%.
292 Very low sporulation levels were observed in the strains of the *Lecanicillium* sp., thus we were
293 not able to obtain a highly concentrated suspension, but performed the test at 1×10^7 conidia/mL.
294 At this concentration, no strain of *Lecanicillium* sp. showed mortality with a statistically
295 significant difference from the control (F=2.9, p < 0.023) (Fig. 2C and *Data S1*), although all
296 strains showed some degree of infectivity (mycosis on the mummified mite).
297 To compare the fungal strains' speed to kill *T. merganser* adult females, we calculated the time it
298 took to reach 50% of mortality (LT_{50}) at $1x10^8$ conidia/mL with strains that showed significant
299 differences in mortality, compared to the control (Table 1). In this preliminary assay, the five *M.*
300 *anisopliae* s.l. strains that caused 100% mortality to *T. merganser* also showed the fastest
301 mortality times (Ma002, Ma005, Ma034, Ma004, and Ma014). Of the *B. bassiana* strains, Bb016
302 exhibited the shortest LT_{50}, followed by Bb005 and Bb014. These are also the three *B. bassiana*
303 strains that caused the highest mortality to *T. merganser*. The chi-square, fiducial limits (FL) and
304 slope (m) from Probit analysis are shown in Table 1.
305 **LC_{50} & LT_{50} of the most virulent strains**
306 We selected the six strains of *M. anisopliae* s.l. (Ma002, Ma003, Ma004, Ma005, Ma014 and
307 Ma034) and the one of *B. bassiana* (Bb016) that caused 90%-100% mortality to *T. merganser*
308 and showed the lower LT_{50} in the mass screening assay to evaluate their virulence with serial
309 doses for seven days and with more replicates (*Data S1*). The doses required to cause mortality
310 to 50% of a population (LC_{50}) were determined and the LT_{50} was recalculated. The most virulent
311 strains were Ma034, Ma005, Ma003, Bb016 and Ma002, with LC_{50} values around
312 10^6 conidia/mL (1.7-3.2 x 10^6, respectively) (Table 2); which also have the lowest LT_{50}. The
313 Ma004 and Ma014 strains had LC_{50} values above 10^7 conidia/mL and the highest LT_{50}. The chi-
314 square, fiducial limits (FL) and slope (m) from Probit analysis for LC_{50} are shown in Table 2.
315 Taken together, the LT_{50}, and LC_{50} data allowed us to identify Ma034 and Ma005 as the most
316 aggressive strain towards *T. merganser* adult females followed by Ma003, Bb016 and Ma002.
317 The ITS of these seven strains were sequenced and we confirmed the identification of Bb016 as
318 *B. bassiana* (Table 3). According with BLASTn hits, ITS from three strains of *M. anisopliae* s.l.
319 (Ma002, Ma003 and Ma014) showed the highest similarity with *M. anisopliae* and three with *M.*

320 *robertsii* (Ma004, Ma005 and Ma034) (Table 3). The phylogenetic analysis confirmed that these
321 six strains belong to the so-called PARB clade of *M. anisopliae* (Table 3, *Figure S1*). Altough
322 the ITS information is not enough to distinguish between the species of PARB clade (Bischoff et
323 al., 2009; Rehner and Kepler, 2017); the ITS alignment showed that Ma004, Ma005 and Ma034
324 have a different number of adenines in a short stretch of this nucleotide, comparing with Ma002,
325 Ma003 and Ma014 (*Figure S2*). That difference is also present in other *M. robertsii* ITS
326 sequences from the Genbank or UNITE databases, including sequences from the reference
327 strains ARSEF 2575 and ARSEF 23, compared to *M. anisopliae* sequences from ARSEF-7487,
328 JEF-290, BRIP 53293, BRIP 53284.

329

330 **Infectivity on eggs**
331 We recorded that seven *Metarhizium anisopliae* s.l. strains (Ma002, Ma003, Ma004, Ma005,
332 Ma008, Ma014 and Ma034) and two *B. bassiana* strains (Bb016 and Bb017) were infectious to
333 *T. merganser* eggs laid as an additional observation during the screening assay (Fig. 2f and
334 Fig.3). Most of these strains also showed a high mortality to the adult females. Thus, we
335 developed an *in vitro* egg infectivity assay (Fig. 2g).
336 All of the tested strains showed mycosis on the eggs, although four strains (Ma002, Ma003,
337 Bb016 and Bb017) did not show statistically significant differences compared to the natural
338 mortality in the control group (F = 14.83, p < 0.0001) (Fig. 4 and *Data S3*). However, the Ma014
339 and Ma034 strains showed a high infection level, reaching 71.7 and 81.7%, respectively (*Data
340 S3*). The data of the Ma004, Ma005 and Ma008 strains were excluded from the analysis due to
341 their high variability. This analysis points to Ma034 as the most promising strain since it is the
342 most virulent to adult females with 100% mortality, has the lowest LC_{50} and low LT_{50} value, and
343 is highly infectious to eggs.

344

345 ## Discussion
346 We confirmed the presence of the emergent pest *T. merganser* in papaya orchards in a new
347 region of southeastern Mexico and proposed biological alternatives using entomopathogenic
348 fungi for its control. Through taxonomical identification, we found two *Tetranychus* species in
349 the orchards sampled in this study: *T. urticae* and *T. merganser*. The presence of *T. urticae* was
350 expected, since it is a cosmopolite pest for many crops and it was previously reported in papaya
351 fields (Abato-Zarate et al., 2014; Mena et al., 2017). Although papaya producers have reported
352 that the red spider mite is the predominant pest of this crop in Campeche, the presence and
353 identity of these two *Tetranychus* species had not been confirmed in this region. However, *T.
354 merganser* was previously reported in commercial papaya crops of other regions along the Gulf
355 of Mexico (Valencia-Domínguez et al., 2011, Abato-Zarate et al., 2014; Monjarrás-Barrera et al.,
356 2017). The presence of high densities of *T. merganser* in papaya orchards in the north and
357 southwest of Campeche suggests that the mites could be more widespread than currently
358 recorded. They may be found throughout the entire Yucatan Peninsula and other surrounding
359 regions. Furthermore, its persistence measured over two years confirms its status as an emerging

360   plague and highlights the importance of seeking strategies to control it in the field. The presence
361   of this emergent pest in other tropical crops must be studied to determine its impact there.To find
362   a biological alternative for the control of *T. merganser* and to reduce the use of chemical
363   acaricides, responding to the requests of local papaya producers, we evaluated thirty
364   entomopathogenic fungal strains from our collection: *M. anisopliae* s.l., *B. bassiana,* and
365   *Lecanicillium* sp. We compared these with *H. thompsonii* var. *sinematosa* isolated from a mite.
366   The CHE-CNRCB 377 strain did not show any infectivity towards *T. merganser*, despite *H.*
367   *thompsonii* var. *sinematosa* being previously described as a potential agent to control mites
368   (Quesada-Sojo and Rivera-Méndez, 2016), including Tetranychidae (Rosas, 2003); this may be
369   due to CHE-CNRCB 377 being isolated from a mite of a different family (Acari: Eriophyidae)
370   (Rosas-Acevedo and Sampedro-Rosas, 1990; Cabrera et al., 2007).
371   Although *Lecanicillium lecanii* was reported as a fungus with a natural incidence in *Tetranychus*
372   (Chandler et al., 2000), none of our *Lecanicillium* sp. strains showed the potential to be applied
373   in field against *T. merganser*. Our *Lecanicillium* sp. strains caused infection to *T. merganser* with
374   low mortality, in agreement with findings of Chandler et al. (2005). In addition, they have low
375   sporulation levels and the predicted LT_{50} is very long. All of these characteristics exclude them
376   from mass production and use in the field.
377   However, despite not being isolated from mites under natural conditions (Wekesa et al., 2015),
378   all of the *Metarhizum anisopliae* s.l. strains and many of the *B. bassiana* strains that were tested
379   showed significant lethality to adult females. This indicates that *T. merganser* is susceptible to
380   these entomopathogenic fungi. Previous reports have shown the infectivity of these two species
381   against other members of the Tetranychidae family, mainly *T. urticae* (Draganova and Simova,
382   2010; Seiedy et al., 2010; Wekesa, 2015; Habashy et al., 2016; Shin et al., 2017). This infectivity
383   is feasible due to the artificial interaction between host-pathogen that may be established
384   between this fungal group (Hyphomycetes) and Acari at high conidial concentrations. The
385   surface structure and chemical composition of the host cuticula likely determines the success of
386   the interaction (Pedrini et al., 2007). Since the mites have similar protection barriers as insects
387   (Chandler et al., 2000), the entomopathogenic fungi could infect them through similar
388   mechanisms.
389   Our data show that all the *M. anisopliae* s.l. tested strains and six of *B. bassiana* were infectious
390   to adult females of *T. merganser*. Additionally, *T. merganser* at this stage was highly susceptible
391   (95-100% of mortality and short LT_{50} values) to six strains of *M. anisopliae* s.l. and one of *B.*
392   *bassiana*. Variations in the effectiveness between strains of a same fungal specie is a common
393   phenomenon, possibly due to the high genetic and metabolic diversity between strains (Wang
394   and St. Leger, 2005; Xiao et al., 2012; Pattemore et al., 2014; Wang et al., 2016; Zhang et al,
395   2021). In comparison with other reports, the performance of our assay after adding the fungus by
396   immersing the leaf-disks was comparable to a spray application (Wekesa et al., 2005; Zhang et
397   al., 2014). The strains tested here were more lethal and virulent (lower LC_{50}) against *T.*
398   *merganser* than other strains of both fungi tested against *T. urticae* (Bugeme et al., 2014). Other
399   studies show similar levels of virulence for *T. urticae* than our strains for *T. merganser* (Tamai et

400 al., 1999; Alves et al., 2002; Wekesa et al., 2005; Chandler et al., 2005; Habashy et al., 2016).
401 We found that more strains of *M. anisopliae* s.l. than of *B. bassiana* were highly infective to *T.*
402 *merganser*, however, other studies have found *B. bassiana* strains more virulent to *T. urticae*
403 than all *Metarhizium* strains reported (Seiedy et al., 2010). Thus, strains of both fungi species
404 seem to be highly infective against *Tetranychus* species.
405 Finally, since egg control is important to decrease or avoid future outbreaks in the field, we
406 evaluated the infectivity of our strains on eggs. Entomopathogenic fungi have been reported to
407 have the ability to infect insects in all stages, including eggs (Hajek and St. Leger, 1994), which
408 is also the case for mites. The infection process in the eggs is similar to that which occurs in the
409 body of arthropods, without the development of blastospores inside the egg as occurs in the
410 hemocele of insects (Zhang et al., 2014). Several studies have evaluated the susceptibility of *T.*
411 *urticae* eggs to different entomopathogenic fungal species. It has been found that the egg
412 response varies from extremely lethal to non-infectious, depending on the strain (Shi and Feng,
413 2004; Bugeme et al., 2014; Zhang et al., 2014; Dogan et al., 2017). Although nine of our strains
414 showed infectivity towards *T. merganser* eggs, only two *Metarhizium* strains (Ma014 and
415 Ma034) showed consistently high infectivity (above 70%) towards eggs. This ovicidal activity is
416 relevant, because the pest cannot regenerate if a high percentage of eggs die in the population.
417 The use of strains which combine a high virulence to adult females with a high egg mortality,
418 would allow a more effective control of *T. merganser* in crops.
419 Our data show that five strains (Ma034, Ma005, Ma003, Bb016 and Ma002) are the most
420 virulent to adult females (lowest LC_{50}). Ma034 and Ma005 are the most aggressive (100%
421 mortality and lowest LC_{50} & LT_{50}). Additionally, Ma034 showed consistently high infectivity
422 towards eggs, which allowed us to select it as the most promising for field testing. Although
423 Bb016 was among the most lethal strains with a low LC50 value, the low infectivity on eggs is
424 an important parameter for its efficacy in the field, so its use could be combined with other egg-
425 infecting strains.

426 The most aggressive *Metarhizium* strains described in this work belong to the PARB clade. *M.*
427 *anisopliae* s.l. is a complex of 12 species, among them the clade informally called PARB that
428 includes *M. pingshaense*, *M. anisopliae*, *M. robertsii* and *M. brunneum*, only distinguishable
429 with a multilocus phylogenetic analysis (Bischoff et al., 2009; Rehner & Kepler, 2017). These
430 type species represent the core of the *M. anisopliae* complex, are the most frequently isolated
431 from insects and soils, have a wide host range and a global distribution (Bischoff et al., 2009;
432 Rehner & Kepler, 2017). It is important to note that the strains described here as infective to *T.*
433 *merganser* are native to the region where this emergent pest has been reported, reducing the risks
434 to other non-target arthropods.
435

Conclusions

437 *T. merganser* is an emerging pest present in the papaya crops of Campeche, Mexico, in addition
438 to *T. urticae*. Adult females and eggs of *T. merganser* are highly susceptible to some strains of
439 the entomopathogenic fungi *M. anisopliae* (PARB clade) and *B. bassiana*. Five strains identified

440   here are as the most infective and virulent against *T. merganser* have good potential to be used in
441   integrated management programs to control this pest. The most aggressive strain, Ma034, also
442   infective for eggs, is the most promising to be tested in papaya fields. The beneficial effects of
443   these entomopathogenic fungi on the economic impact of this pest or on the use of toxic
444   chemicals remains to be addressed.
445

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449   orchards, and Ph. D. Victor Hugo Quej-Chi for help with Figure 1.
450

Supplementary files

452   **S1** Values of mortality in screening assay for *Metarhizium*, *Beauveria* and *Lecanicillium* against
453   *T. merganser* (raw data)
454   **S2** Mortality values for the most lethal entomopathogenic strains against adult female T.
455   merganser and LC50 analysis.
456   **S3** Infectivity values on *T. merganser* eggs susceptible to *Beauveria* and *Metarhizium* strains.

457   **Figure S1**: This file include: the phylogenetic tree comparing the ITS sequences of *M.*
458   *anisopliae* s.l. from this work versus ITS of *Metarhizium* spp. reference strains.

459   **Figure S2**: This file include: the alignment showing a difference in the adenine number between
460   the ITS sequences of M. *anisopliae* and *M. robertsii*.
461

Figure/Table legends
463   **Figure 1.**
464   Title: *Tetranychus* spp. identified in papaya orchards in the state of Campeche, Mexico
465   Legend:
466   Presence of *Tetranychus* species found in the papaya orchard during the 2017-2018 season
467   (table); sampling sites were located in the north and south of the state where the main
468   agricultural areas were located (circles). Proximity to nature reserves and average annual climate
469   are shown: Calakmul (1), Petenes (2) and Laguna de Términos (3); (T° C), relative humidity
470   (RH). The presence of *T. merganser* in this work confirms its distribution along the Gulf of
471   Mexico (black areas).
472

473   **Figure 2.**
474   Title: Identification of *Tetranychus merganser* and bioassays
475   Legend:
476   (a) *T. merganser* showing bright red females and pale yellowish males while *T. urticae* (b) their
477   females are greenish, these females were used in the mass screening for bioinfectivity; (c) *T.*
478   *merganser* (male) showing aedeagus knob resembles the head and neck of a goose (box); d) *T.*
479   *urticae* (male) showing the aedeagus knob of has an anterior projection acute and a rounded

480   shape in posterior projection (box); d) eggs infected in screening assay, e) eggs on mesh (130
481   μm) for the infectivity bioassay, and f) egg infected and stained with lactophenol cotton-blue.
482
483   **Figure 3.**
484   Title: Percentage mortality of *T. merganser* by different strains of entomopathogenic fungi (1 x
485   10^{8} conidia/ml)
486   Legend: a) *M. anisopliae* s.l., b) *B. bassiana*, and c) *Lecanicillium* sp. (1×107 conidia/mL).
487   Asterisk: strains that were infective towards eggs. Columns with different letters are significantly
488   different among strains based on Tukey´s test (p<0.05) after one-way ANOVA analysis. Bars:
489   standard error.
490
491   **Figure 4.**
492   Title: Infectivity on eggs of *T. merganser* of six strains of entomopathogenic fungi.
493   Legend: Columns with different letters are significantly different among strains based on
494   Tukey´s test (p<0.05) after one-way ANOVA analysis. Bars: standard error.
495
496   **Table 1**. Estimated lethal time (LT_{50}) of fungal isolates infecting adult females of *T. merganser*
497   Bb: *Beauveria bassiana*, Ma: *Metarhizium anisopliae*, se: Standard error, FL: fiducial limits
498   95%, X^{2}: chi-square, m: slope of regression line.
499
500   **Table 2**. Median lethal concentration (LC_{50}) of more lethal strains for *T. merganser* adult
501   females
502   X^{2}: chi-square, m: slope of regression line ± standard error; FL: fiducial limits 95%
503
504   **Table 3.** Highly virulent strains to *T. merganser* selected from screening and BLASTN data
505   *Collected by Comité Estatal de Sanidad Vegetal Campeche (Cesavecam). E: expect value.
506   [1]Unidentified species
507
508

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Figure 1

Tetranychus spp. identified in papaya orchards in the state of Campeche, Mexico

Presence of *Tetranychus* species found in the papaya orchard during the 2017-2018 season (table); sampling sites were located in the north and south of the state where the main agricultural areas were located (circles). Proximity to nature reserves and average annual climate are shown: Calakmul (1), Petenes (2) and Laguna de Términos (3); (T°C), relative humidity (RH). The presence of *T. merganser* in this work confirms its distribution along the Gulf of Mexico (black areas).

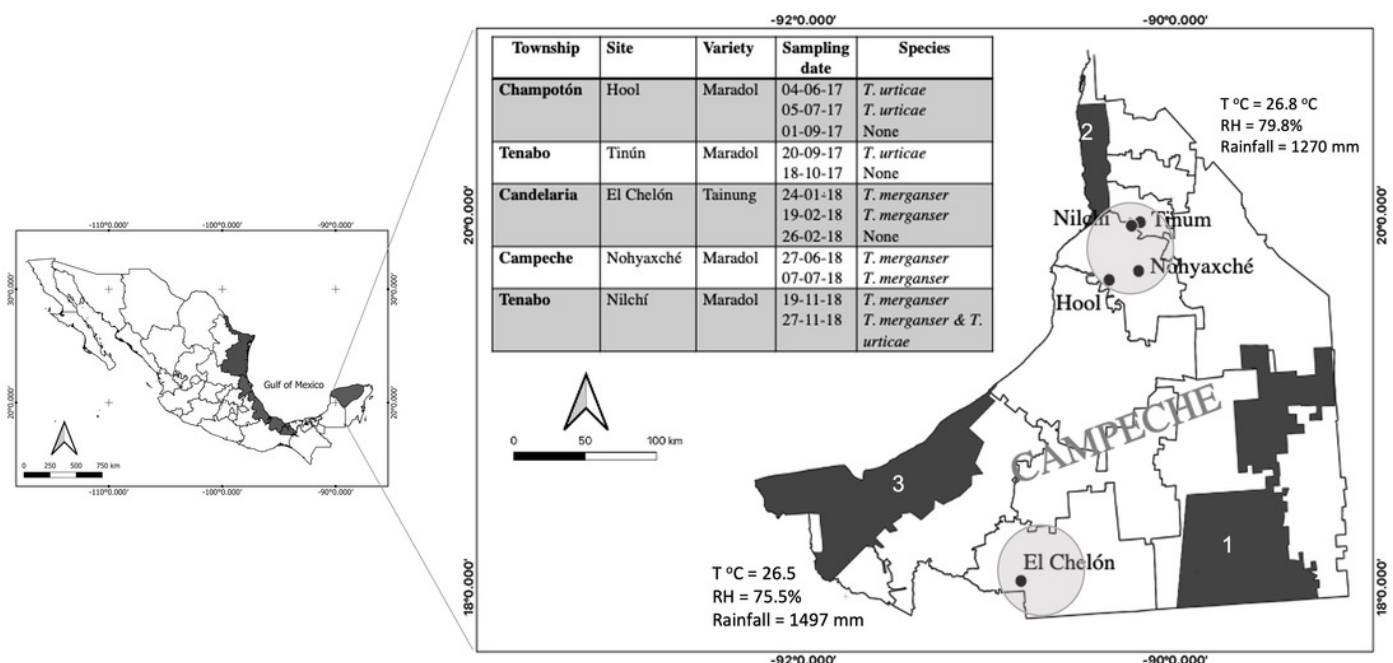



Figure 2

Identificationof*Tetranychus merganser*and bioassays

(a) *T. merganser* showing bright red females and pale yellowish males while *T. urticae* (b) their females are greenish, these females were used in the mass screening for bioinfectivity; (c) *T. merganser* (male) showing aedeagus knob resembles the head and neck of a goose (box); d) *T. urticae* (male) showing the aedeagus knob of has an anterior projection acute and a rounded shape in posterior projection (box); d) eggs infected in screening assay,e) eggs on mesh (130 µm) for the infectivity bioassay, and f) egg infected and stained with lactophenol cotton-blue.

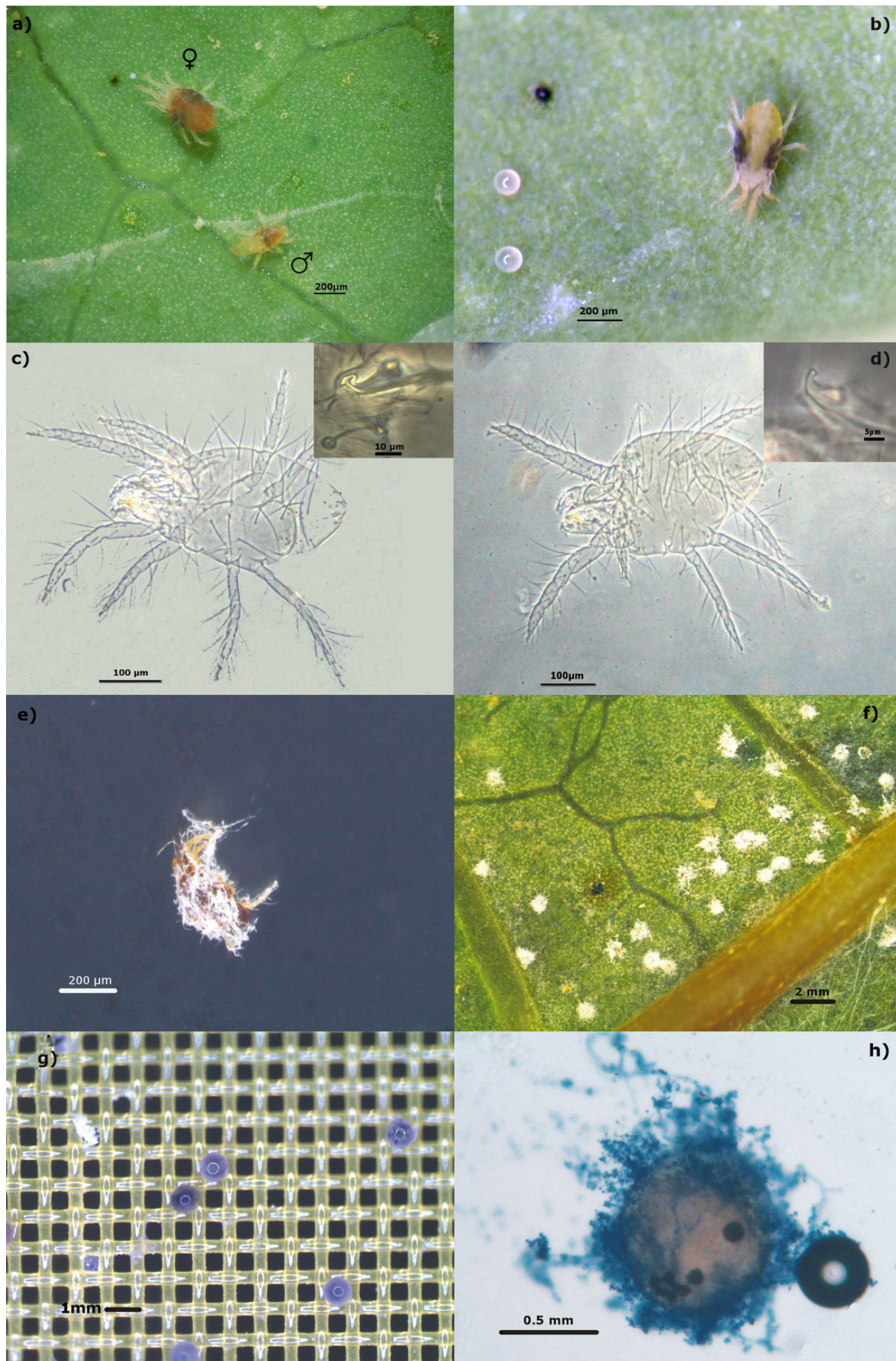



Figure 3

Percentage mortality of*T. merganser*by different strains of entomopathogenic fungi (1 x 10^{8}conidia/ml)

a)*M. anisopliae* s.l., b)*B. bassiana*,and c)*Lecanicillium*sp. (1×107conidia/mL). Asterisk: strains that were infective towards eggs. Columns with different letters are significantly different among strains based on Tukey´s test (p<0.05) after one-way ANOVA analysis. Bars: standard error.

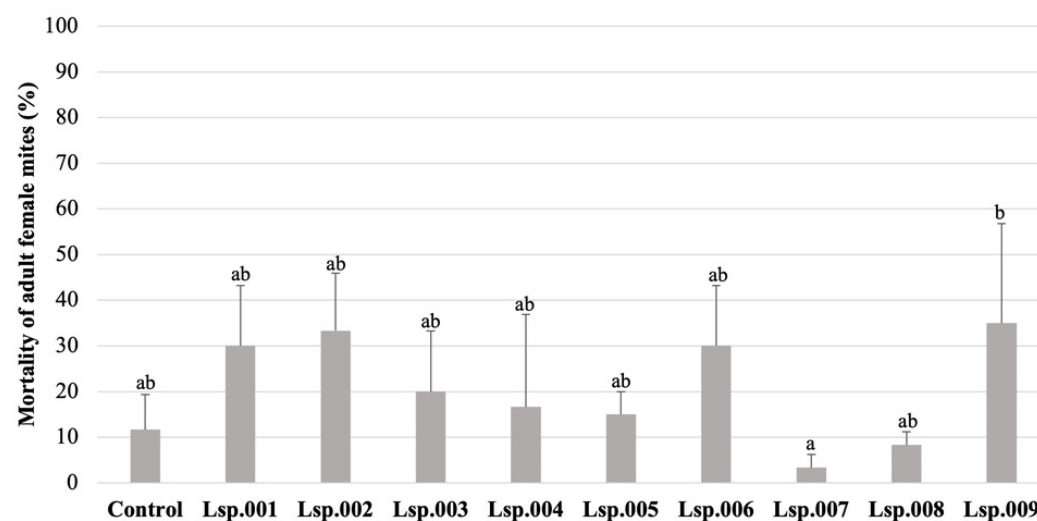
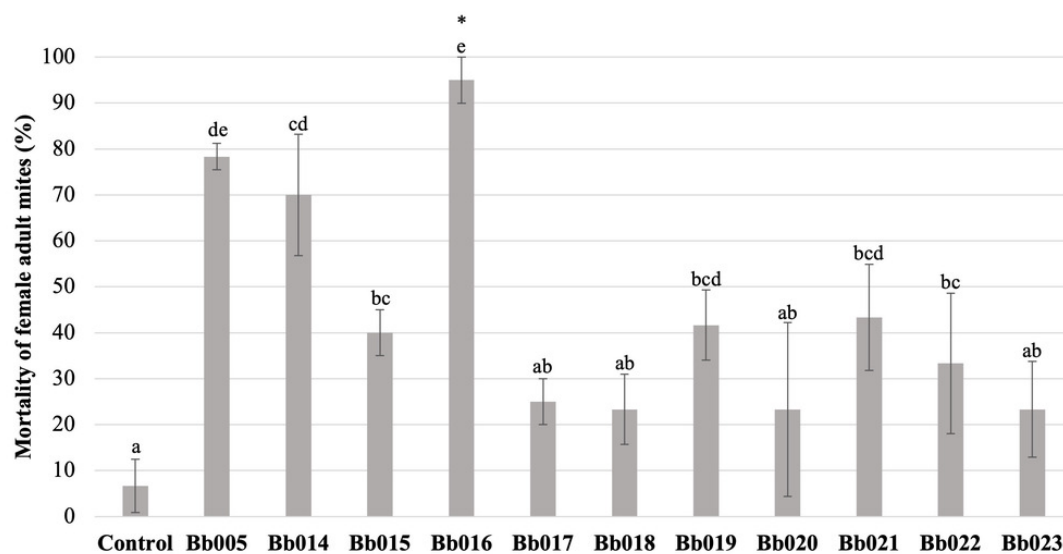
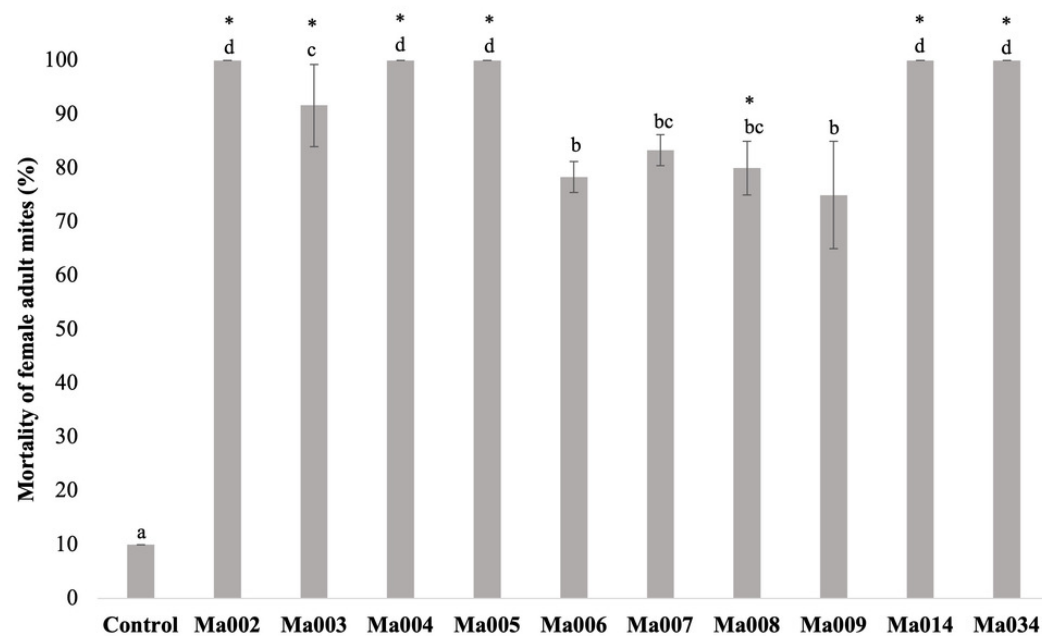
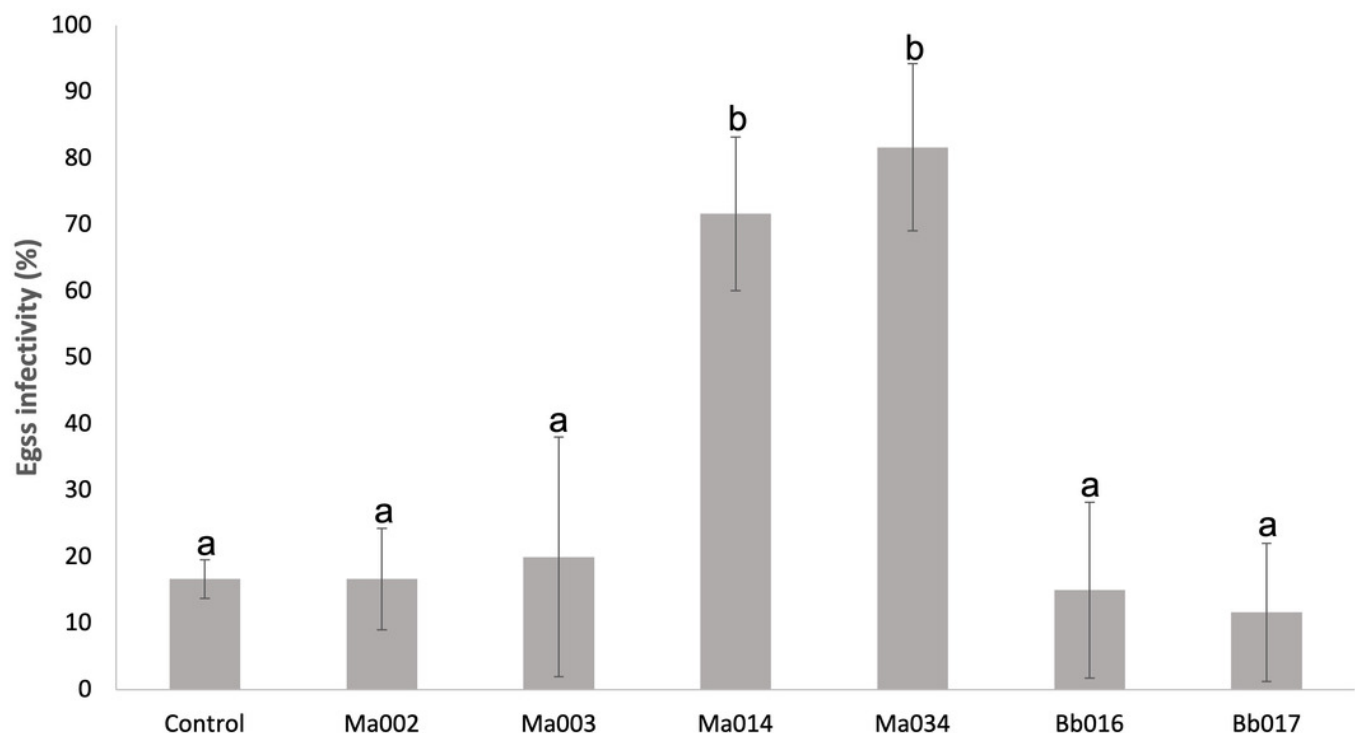



Figure 4

Infectivity on eggs of*T. merganser*of six strains of entomopathogenic fungi.

Columns with different letters are significantly different among strains based on Tukey´s test (p<0.05) after one-way ANOVA analysis. Bars: standard error.

Estimated lethal time (LT$_{50}$) of fungal isolates infecting adult females of *T. merganser*

Bb: *Beauveria bassiana*, Ma: *Metarhizium anisopliae*, se: Standard error, FL: fiducial limits

95%, <!--[if !msEquation]--> <!--[if !vml]--> <!--[endif]--><!--[endif]-->**:** chi-square, m: slope

of regression line.

2 **Table 1**. Estimated lethal time (LT_{50}) of fungal isolates infecting adult females of *T. merganser*

Strain	LT_{50} (days) ±se	FL_{lower}	FL_{upper}	X^2	m ± se
Bb005	4.79 ± 0.24	4.37	5.33	0.92	0.93 ± 0.25
Bb014	5.05 ± 0.40	4.54	6.24	0.89	0.89 ± 0.24
Bb015	5.93 ± 0.49	5.47	7.64	1.08	1.08 ± 0.57
Bb016	3.96 ± 0.20	3.55	4.34	0.96	0.96 ± 0.25
Bb019	6.38 ± 0.92	5.66	10.00	0.95	0.94 ± 0.48
Bb021	6.29 ± 1.44	5.51	16.63	0.88	0.88 ± 0.38
Ma002	3.24 ± 0.16	2.88	3.51	0.46	1.13 ± 0.49
Ma003	4.60 ± 0.29	4.16	5.34	1.21	0.95 ± 0.27
Ma004	3.64 ± 0.20	3.23	4.00	1.00	1.94 ± 0.35
Ma005	3.28 ± 0.19	2.87	3.60	1.26	1.03 ± 0.39
Ma006	4.37 ± 0.26	3.88	4.92	2.39	0.81 ± 0.17
Ma007	3.97 ± 0.29	3.36	4.48	0.99	0.78 ± 0.16
Ma008	4.54 ± 0.26	4.08	5.14	1.56	0.88 ± 0.20
Ma009	5.03 ± 0.23	4.65	5.56	1.96	1.03 ± 0.37
Ma014	3.69 ± 0.18	3.31	4.03	1.98	1.05 ± 0.37
Ma034	3.35 ± 0.18	2.93	3.65	1.80	1.03 ± 0.38

3 Bb: *Beauveria bassiana*, Ma: *Metarhizium anisopliae*, se: Standard error, FL: Fiducial limits 95%, X^2: Chi-square, m: slope of
4 regression line

Median lethal concentration (LC$_{50}$) of more lethal strains for *T. merganser* adult females

X2: chi-square, m: slope of regression line ± standard error; FL: fiducial limits 95%

2
3
4
5 **Table 2**. Median lethal concentration (LC50) of more lethal strains for *T. merganser* adult
6 females

Strain	LC_{50} conidia/ml	FL_{lower}	FL_{upper}	X2	m ± se	LT50 ± se
Ma034	1.73E+06	7.94E+05	3.85E+06	2.38	1.04±0.17	3.70±0.20
Ma005	1.97E+06	9.13E+05	4.34E+06	2.22	1.03±0.17	3.68±0.19
Ma003	2.32E+06	1.03E+06	5.56E+06	2.29	1.04±0.17	3.94±0.24
Bb016	2.62E+06	1.32E+06	5.35E+06	1.11	1.39±0.26	3.91±0.22
Ma002	3.21E+06	1.53E+06	6.96E+06	1.88	1.20±0.21	3.93±0.21
Ma004	1.26E+07	5.13E+06	4.49E+07	0.78	0.87±0.17	4.72±0.37
Ma014	5.27E+07	1.54E+07	9.13E+08	1.20	0.70 ±0.16	6.11 ±0.41

9 X^2: Chi-square, m: slope of regression line ± standard error; FL: Fiducial limits 95%
10

Highly virulent strains to *T. merganser* selected from screening and BLASTN data

*Collected by Comité Estatal de Sanidad Vegetal Campeche (Cesavecam). E: expect value.

[1]Unidentified species

Table 3. Highly virulent strains to *T. merganser* selected from the screening and BLASTN data

Strain	Source	Geolocation	Molecular identification	Score (Bits)	E value	Genbank accession	Clade
Bb016	Soil	18°35'35.2"N 90°01'58.2"W	*Beauveria bassiana*	480	2e-134	OM490017	
Ma002*	Chrysomelinae[1]	unknown	*Metarhizium anisopliae*	904	0.0	OM490011	PARB
Ma003*	Spodoptera larvae[1]	unknown	*Metarhizium anisopliae*	713	0.0	OM490012	PARB
Ma004	Cercopidae	18°32'50.5"N 89°54'28.8"W	*Metarhizium robertsii*	558	1e-157	OM490013	PARB
Ma005	Soil	18°33'06.4"N 89°54'20.5"W	*Metarhizium robertsii*	671	0.0	OM490014	PARB
Ma014*	Coccineliidae[1]	unknown	*Metarhizium anisopliae*	743	0.0	OM490015	PARB
Ma034*	Cercopidae[1]	18°32'46.8"N 89°54'45.6"W	*Metarhizium robertsii*	564	3e-159	OM490016	PARB

*Collected by Comité Estatal de Sanidad Vegetal Campeche (Cesavecam). E: expect value. [1]Unidentified species