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Potential for entomopathogenic fungi to control *Triatoma dimidiata* (Hemiptera: Reduviidae), a vector of Chagas disease in Mexico

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ABSTRACT

Introduction: The use of entomopathogenic fungi to control disease vectors has become relevant because traditional chemical control methods have caused damage to the environment and led to the development of resistance among vectors. Thus, this study assessed the pathogenicity of entomopathogenic fungi in *Triatoma dimidiata*. **Methods***:* Preparations of 10^8 conidia/ml of *Gliocladium virens*, *Talaromyces flavus*, *Beauveria bassiana* and *Metarhizium anisopliae* were applied topically on *T. dimidiata* nymphs and adults. Controls were treated with the 0.0001% Tween-80 vehicle. Mortality was evaluated and recorded daily for 30 days. The concentration required to kill 50% of *T. dimidiata* (LC_{50}) was then calculated for the most pathogenic isolate. **Results***:* Pathogenicity in adults was similar among *B. bassiana*, *G. virens* and *T. flavus* ($p>0.05$) and differed from that in triatomine nymphs ($p=0.009$). The most entomopathogenic strains in adult triatomines were *B. bassiana* and *G. virens*, which both caused 100% mortality. In nymphs, the most entomopathogenic strain was *B. bassiana*, followed by *G. virens*. The native strain with the highest pathogenicity was *G. virens*, for which the LC_{50} for *T. dimidiata* nymphs was 1.98×10^8 conidia/ml at 13 days after inoculation. **Conclusions**: *Beauveria bassiana* and *G. virens* showed entomopathogenic potential in *T. dimidiata* nymphs and adults. However, the native *G. virens* strain presents a higher probability of success in the field, and *G. virens* should thus be considered a potential candidate for the biological control of triatomine Chagas disease vectors.

Keywords: Biological control. Chagas disease. Entomopathogenic fungi. *Triatoma dimidiata*.

INTRODUCTION

Chagas disease is one of the major neglected tropical diseases transmitted by insect vectors. It causes high morbidity and mortality, reaching levels comparable to those observed for human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) in the Latin American region[1]. However, the current extent of globalization of the disease is evident in the 300,000 to 1,000,000 cases present in the United States of America[2], and the disease has expanded into Canada, Europe, Australia and Japan[3], as well. Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi* and is transmitted by hematophagous bugs of the order Hemiptera, family Reduviidae, subfamily Triatominae. Currently, 147 species are included in this group[4,5], but species from only three genera, *Triatoma, Rhodnius* and *Panstrongylus*, are widely distributed in endemic areas in the Americas, from Mexico through Argentina and Chile[6], and serve as the main vectors of *T. cruzi* in domestic animals and humans.

In Mexico, Chagas disease has been reported in all 31 states and in the capital city[7]. Thirty-three triatomine species have been identified, of which 28 are endemic and 22 have been reported to be infected with *T. cruzi*[8-12]. Based on their domiciliary habits, the most important of these are *Triatoma longipennis*, *Triatoma mazzotti*, *Triatoma pallidipennis*, *Triatoma picturata*, *Triatoma barberi*, *Triatoma mexicana*, *Triatoma gerstaeckeri* and *Triatoma dimidiata*[7,13]. *Triatoma dimidiata* has a wide geographical distribution and can be found from Mexico to Northern South America; it is one of the most important vectors of Chagas disease in Central America and southern Mexico[6,14].

No vaccine is currently available for Chagas disease, and the existing drug treatment has limited effectiveness and undesirable side effects; thus, anti-vectorial interventions are the main strategy for the control of this disease. These interventions include improvements in housing conditions and vector control through indoor insecticide spraying[15] using several synthetic carbamates, organophosphates and pyrethroids[16-18]. However, in addition to adverse environmental effects of these substances, an increase in the numbers of field vector populations resistant

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to insecticides has been documented, making insecticide-based control strategies inefficient[17-23]. Thus, new alternatives are urgently needed for use in integrated vector control strategies.

Several experiments have documented the efficacy of entomopathogenic fungi, such as *Beauveria bassiana* and *Metarhizium anisopliae*, in the control of Chagas disease vectors under laboratory[24-28] and field conditions[29-32]. The first barrier to the penetration of contact insecticides is the insect cuticle, and an increase in the thickness of the *Triatoma infestans* cuticle was found to be associated with the resistance of this insect to pyrethroids. A strain of *B. bassiana* adapted to grow more easily on insect-like hydrocarbons used in attraction-infection traps, which combine CO_2 with the fungus, has been reported to cause 52.4% mortality in pyrethroid-resistant insects in experimental houses and rural human dwellings. This indicates that the capacity of entomopathogenic fungi to degrade the cuticle of insects is an advantage that could be exploited for the control of insecticide-resistant triatominae[29].

Environmental factors may affect the efficiency of these fungi[26], but native fungal strains, which are adapted to local conditions, may exhibit better performance. We have isolated several entomopathogenic fungal strains from communities near Tapachula, Chiapas, in southern Mexico, with the purpose of developing bioinsecticides for the control of disease vector insects. This study assessed the pathogenicity of native *Gliocladium virens* (Miller) Giddens and Foster and *Talaromyces flavus* (Klöcker) Stolk and Samson in *T. dimidiata* nymphs and adults, compared with *B. bassiana* (Balsamo) Vuillemin and *M. anisopliae* (Metschnikoff) Sorokin, which are pathogenic fungi used in the control of agricultural plagues.

METHODS

Triatomines

Triatoma dimidiata nymphs and adults were collected in 17 communities in the foothills near Tapachula, Chiapas, Mexico (15º 03´-14º 13´ N and 92º 26´-92º 29´ W) via active searches in peridomestic sites. The collected insects were divided into two groups; one group was used for pathogenicity bioassays, while the other group was reared in the laboratory at 27±1ºC under 70±2% relative humidity. First-generation insects raised in the laboratory were used to determine lethal concentrations of the studied fungi. One week before each bioassay, triatomines were fed with rabbit blood. Two days before the bioassay, all triatomines were disinfected by a two-minute treatment with a 0.5% sodium hypochlorite solution. The insects were washed three times with sterile triple-distilled water and dried.

Fungal strains

Four fungal strains were used in the bioassays. *Beauveria bassiana* LBIH-048 was isolated from the *coffee bug* of the sorghum pest *Oebalus mexicana* Sailer, and *M. anisopliae sensu lato* LBIH-033 was isolated from *white grubs* of *Phyllophaga* spp. These fungal strains were obtained from the collection of the Center for Research and Advanced Studies (CINVESTAV, Irapuato, Mexico). The native strains *T. flavus* LBIH-111 and *G. virens* LBIH-116 were isolated from *Anopheles albimanus* larval breeding sites on the coastal plain of Chiapas, Mexico (14º 43´-14º 52´ N and 92º 26´-92º 33´ W) in 2007 and were obtained from the collection of entomopathogenic fungi of the Laboratory of Biocontrol of Disease Vector Insects of the National Institute of Public Health (INSP, Chiapas, Mexico). All of the fungal strains were deposited in the collection of entomopathogenic fungi (REDBIO) at the laboratory of bioinsecticides at CINVESTAV in Irapuato, Mexico.

The fungi were cultured on Sabouraud dextrose agar medium (SDA, Bioxon®, Becton Dickinson de Mexico) at 27±1°C until conidia were visible, which occurred within approximately 21 days. Conidial suspensions were prepared in sterile distilled water with 0.0001% Tween-80. The viability of the fungi was assessed by measuring conidial germination on SDA medium under optimal conditions[33]. Conidial suspensions demonstrating more than 90% germination were used in the bioassays. Conidial concentrations were estimated using a Neübauer camera (Hausser Scientific).

Pathogenicity bioassays

Groups of field-collected insects, consisting of ten nymphs (different instars) and ten adults of *T. dimidiata*, were treated via topical application of 3 and 5ml, respectively, of a fungal suspension consisting of 10^8 conidia/ml of *B. bassiana*, *G. virens*, *T. flavus*, or *M. anisopliae* in 0.0001% Tween-80; the fungal suspension was placed on the surface of the cuticle using a micropipette[33]. Control insects were treated with 0.0001% Tween-80 without conidia. Treated triatomines were placed in containers within an environmental chamber (Thermo Electron Corporation Model 818) at 27±2°C and 75±5% relative humidity (RH) with a 10h light:14h dark photoperiod. The deaths of insects were recorded daily for 30 days after treatment. Each bioassay consisted of 10 insects per fungal strain and 10 insects in a control group. The number of repetitions of each assay varied from three to six, depending on the availability of insects.

The percentage of insects exhibiting spore production was obtained using a stereo microscope to quantify the number of dead individuals with spores in relation to the dead individuals without spores. Mortality for each fungal treatment was measured as the average time (days) between a fungal application and the death of an insect; only triatomines with spores were included in the counts.

Lethal concentration (LC_{50})

Given the high pathogenicity of *G. virens* observed in the bioassays using nymphs and adults, we chose to evaluate the concentration of this fungus required to kill 50% (LC_{50}) of *T. dimidiata* nymphs. Six concentrations, $5x10^8$, $1x10^8$, $5x10^7$, $1x10^7$, $5x10^6$ and $1x10^6$ conidia/ml in 0.0001% Tween-80, were tested on groups of 20 insects, with three repetitions for each conidial concentration and for a control group, for a total of 420 triatomines. The control groups were treated with 0.0001% Tween-80 solution without conidia. Daily observations of mortality were carried out for 30 days, as indicated above.

Statistical analysis

A generalized linear model (GLM) and variance analysis with multiple mean comparisons using the least significant difference (LSD) test were used to compare mortality, the percentages of insects exhibiting spore formation and the average number of days until death in triatomines with different treatments[34]. The deaths recorded with different fungal concentrations were subjected to probit analysis (EPA, Version 1.5). The mortality curves obtained for the different concentrations of the selected fungal isolate were adjusted to sigmoid curves, for which the results of goodness-of-fit tests were verified using chi-squared tests. The curves were then linearized by probit transformation, and the LC_{50} of the population was calculated using a regression equation, assuming normality[35].

RESULTS

Pathogenicity bioassays

The fungus *B. bassiana* and the native strain *G. virens* exhibited the highest pathogenicity in *T. dimidiata* nymphs, resulting in 73.3% and 35.7% mortality, respectively, at 30 days after treatment. For the *M. anisopliae* and *T. flavus* strains, nymph mortality was 20% and 16.6%, respectively. No deaths were recorded among the controls (**Figure 1**).

It was observed that lower pathogenicity of the fungal strains was related to lower levels of spore formation. The rate of spore-producing infections in dead nymphs was 70% for *G. virens*, 66.7% for *B. bassiana* and 25% for *M. anisopliae*. The *T. flavus* strain did not result in spore-producing infections.

Similarly, *B. bassiana*, which exhibited the greatest pathogenicity, caused mortality more rapidly, as well; the first dead nymph was detected nine days after conidial treatment. *Gliocladium virens* and *M. anisopliae* caused nymphs to die after an average of 17 days, and *T. flavus* caused nymphs to die after 21 days.

Gliocladium virens, the native fungal strain, and *B. bassiana* caused 100% mortality in *T. dimidiata* adults. *Talaromyces flavus* had a 75% mortality rate, and *M. anisopliae* had a 25% mortality rate (**Figure 2**). *Beauveria bassiana* caused adult triatomines to die within 13.6 days, on average, and *G. virens* caused death in only 8.3 days. For *T. flavus* and *M. anisopliae*, adult triatomine mortality was observed after an average of 16 and 24 days, respectively. Fungal development on dead adults was examined; viable conidia were detected on the surface of 88.8% and 50% of cadavers treated with *B. bassiana* and *G. virens* (**Figure 3**), respectively.

The results obtained from the pathogenicity bioassays revealed significant differences among the fungi in terms of the mortality of *T. dimidiata* nymphs and adults compared with the control group. Mortality in the control group was 0% for nymphs and 10% for adults. The pathogenicity of the fungal strains in adults was similar among *B. bassiana*, *G. virens* and *T. flavus* ($p>0.05$) and different from those in nymphs ($F=1.77$; d.f.=53; $p=0.009$). Significant differences in spore formation were observed among the four tested strains ($F=1.84$; d.f.=41; $p=0.04$), whereas no significant difference in the average number of days required to kill triatomines was observed among the strains ($F=0.47$; d.f.=28; $p=0.95$).

In the pathogenicity bioassays, *G. virens* exhibited the highest pathogenicity (mortality rate, sporulation and time to kill) in *T. dimidiata* in both adults and nymphs.

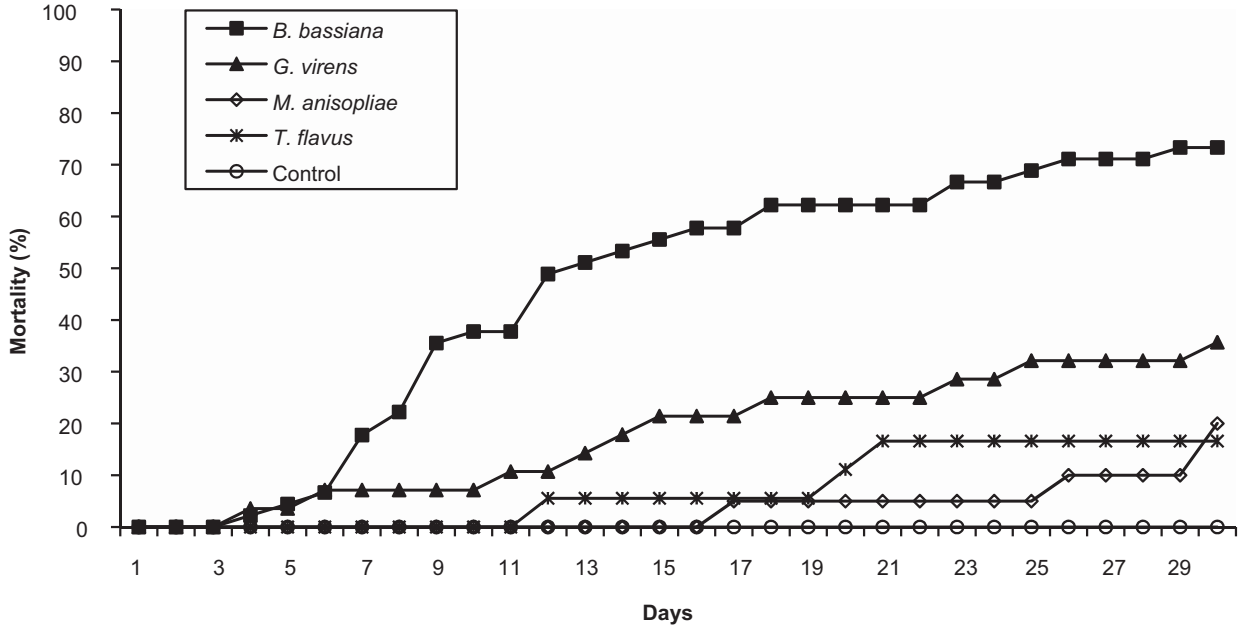

FIGURE 1 - Cumulative mortality of *Triatoma dimidiata* nymphs topically inoculated with fungal suspensions containing 10^8 conidia/ml of *B. bassiana*, *G. virens*, *M. anisopliae* and *T. flavus*. *B.:* Beauveria; *G.:* Gliocladium; *M.:* Metarhizium; *T.:* Talaromyces.

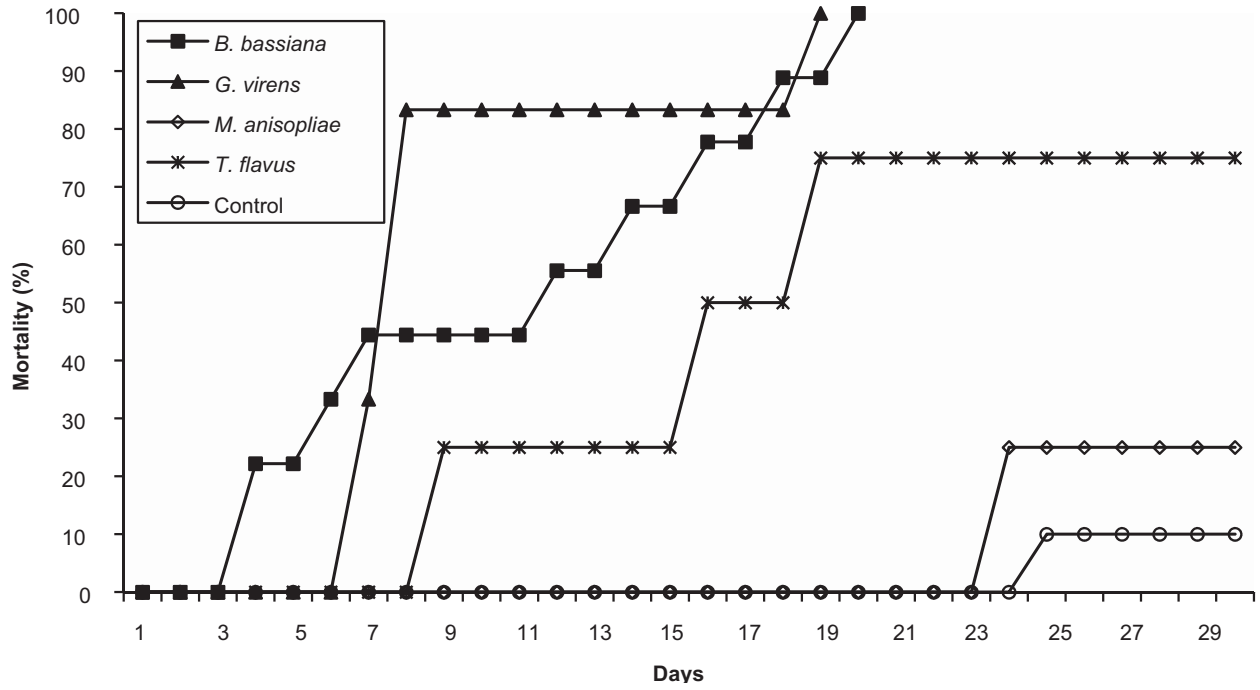

FIGURE 2 - Cumulative mortality of *Triatoma dimidiata* adults topically inoculated with fungal suspensions containing 10^8 conidia/ml of *B. bassiana*, *G. virens*, *M. anisopliae* and *T. flavus*. *B.:* Beauveria; *G.:* Gliocladium; *M.:* Metarhizium; *T.:* Talaromyces.

FIGURE 3 - *Triatoma dimidiata* adult infected with *Gliocladium virens*.

Lethal concentration (LC_{50})

In the bioassays using different concentrations of *G. virens* conidia, the mortality rate among *T. dimidiata* nymphs increased as the concentration of conidia increased. The highest concentration ($5x10^8$ conidia/ml) resulted in 58% mortality after 13 days and 80% mortality at 30 days after inoculation.

Deaths recorded after 13 days were used to determine the LC_{50} using the probit method. A calculated chi-squared value of $\chi^{2=}3.039$ was obtained, and a heterogeneity test revealed no significant heterogeneity ($p>0.05$). Using adjusted values for the observed responses, the LC_{50} of *G. virens* in *T. dimidiata* nymphs was calculated to be $1.98 x10^8$ conidia/ml (1.10-4.68, 95% CI).

DISCUSSION

In this study, *B. bassiana* and the native strain *G. virens* caused the highest pathogenicity in *T. dimidiata* nymphs and adults. Adults were more susceptible to fungal infection than nymphs, most likely because the nymphs lost their conidia during molting after topical inoculation. The triatomines used in this bioassay were collected in the field, and the timing of molting in nymphs therefore could not be controlled. Rocha et al.[36] investigated mass-reared laboratory colonies of *Rhodnius* spp. (third-instar nymphs) treated with *Lecanicillium psalliotae*, *Paecilomyces lilacinus* and *Pochonia chlamydosporia* and calculated that the time until 50% of all infected nymphs died (LT_{50}) was 12.8 days for *Rhodnius neglectus* infected with *P. lilacinus* and 22.6 days for *R. robustus* infected with *L. psalliotae*. In the present study, *G. virens* killed 50% of the exposed population of *T. dimidiata* in 11 days, which is a shorter period of time than was observed by Rocha et al.[36], regardless of the fact that the insects were collected from the field.

It was previously reported that treatment with $1.0 x10^7$ conidia/ml of *B. bassiana* resulted in 100% mortality for *T. infestans* adults at 15 days after inoculation[28]. *Triatoma infestans* mortality following treatment with *B. bassiana* Bb10 conidia grown on CAM medium was 100% in nymphs, and the LT_{50} in nymphs was 7.1 days. In adults, mortality due to

B. bassiana Bb10 conidia was 100%, with an LT_{50} of 4.8 days[26]. These findings suggest that our strain of *B. bassiana* is less pathogenic than strains investigated by others, as our strain causes less mortality over a longer time. These differences could be attributed to variations in triatomine susceptibility. However, little information is available regarding the factors affecting host susceptibility to entomopathogenic fungi. One important factor is the degradation and/or penetration of the initial barrier that must be overcome for successful infection to occur, particularly in relation to the hydrocarbons that constitute the insect epicuticle. The composition of surface lipids has profound consequences that affect the ecological and behavioral characteristics of insects[37]. However, it is more likely that low pathogenicity is an intrinsic characteristic of the fungal strain that we studied[27].

The LC_{50} for *G. virens* infections of *T. dimidiata* nymphs was 1.98 $x10^8$ conidia/ml at 13 days after treatment; this value is higher than those observed in another study using third-instar *T. infestans* nymphs, for which the values of LC_{50} were $7.1x10^5$ for the *B. bassiana* isolate CG 14 and $4.3x10^6$ conidia/ml for the *M. anisopliae* isolate CG 491[38].

A concentration of $2.4x10^6$ conidia/cm^2 of *B. bassiana* was required to kill 50% of *T. infestans* third-instar nymphs at 25 days after inoculation when insects were continuously exposed to conidia. The LC_{50} was significantly higher ($2.0x10^7$ conidia/cm^2) when insects were exposed for 1 h to treated filter paper[39]. In another study, a fungal suspension was used to inoculate *T. infestans* adults; insects were individually submerged in a 5-ml suspension of *B. bassiana* at 10^7 conidia/ml for 7 seconds[28]. The median survival time of *T. infestans* adults inoculated in this fashion was 6.7 days, and 100% mortality occurred by 15 days post treatment[28]. In our study, treatment of *T. dimidiata* adults with a topical application of a suspension of *G. virens* resulted in 100% mortality in an average of 8.3 days, a shorter time than has been reported previously. No previous study has reported *G. virens* pathogenicity in triatomines; until now, it has only been considered to be an antagonist fungus[40]. In recently reported pathogenicity bioassays, the LC_{50} of *G. virens* in *An. albimanus* larvae was found to be 3.3 $x10^5$ conidia/ml in early larval instars and $3.5x10^6$ conidia/ml in late instars[41]. These findings indicate that these insects are more susceptible to *G. virens* compared with *T. dimidiata*.

In preliminary field tests[42], the mortality of *T. infestans* treated with unformulated *B. bassiana* conidia was lower and occurred more slowly in comparison with laboratory tests[38]. *Gliocladium virens* shows greater potential for use in control strategies because, in addition to the fact that native strains are most likely to be successful in the field, this fungus is easily produced in laboratory using artisanal methods[43]. However, further studies are necessary to establish adequate fungal formulations and to determine the most effective method of application under field conditions.

The invasion of domestic and peridomestic triatomines is a major difficulty for Chagas disease control. Periodic seasonal dispersion of adult *T. dimidiata* from sylvatic and peridomestic habitats into houses during the hottest, driest months of the year has been observed in Yucatan, Mexico. While a higher percentage of adults were captured in intradomiciliary locations, the majority of nymphs were collected in peridomiciliary locations. Similar infection rates with *T. cruzi* in insects collected indoors and outdoors[44] highlight the importance of peridomiciliary insects in the risk of transmission of *T. cruzi* to humans[45]. Taking into account environmental factors such as temperature and relative humidity, it has been recommended that *B. bassiana* be applied peridomestically during the rainy season to control peridomestic *T. sordida* and that insecticides be applied during dry periods; a combination of both strategies during both seasons has also been suggested[46]. These strategies could be considered for use with our strain of *G. virens*, a native strain adapted to the environment of southern Chiapas[41]. However, further studies under field conditions are required in the various habitats of *T. dimidiata* to determine appropriate fungal formulations and the most effective application methods, as well residual effects on this insect population.

Although entomopathogenic fungi are more persistent in the environment than other microbial insecticides and convert dead insects into new inoculum sources[47], specific environmental conditions are necessary for fungal germination. This may explain why *T. flavus* did not grow in treated insects in our study. Low humidity has been reported to be responsible for the failure of *B. bassiana* to emerge from the corpses of treated *T. infestans*[38,39].

The rates of *G. virens* infection that were observed (70% in dead nymphs and 50% in dead adults) are encouraging in the context of biological control because all contaminated carcasses exhibited viable conidia. This ensures that under field conditions, the infection could be transmitted to healthy insects via direct passive transfer facilitated by the social behavior of these insects or could be spread by wind. Accordingly, the aggregation of *T. infestans* in small nests facilitates contact between insects and contributes to the transmission of *B. bassiana* in laboratory trials[29]. In addition, under field conditions, dry *B. bassiana* conidia formulations remained viable (88.63%) after three months[29], and its virulence against first and third *T. infestans* instars did not change significantly for up to 5 months under similar conditions[48].

Because *G. virens* is pathogenic in *T. dimidiata* nymphs and adults and because a native strain is most likely to be effective under local field conditions, this species of fungus should be considered a good candidate for the biological control of triatomine vectors of Chagas disease in southern Mexico.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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