

Research Paper

Lethal effects of a Mexican *Beauveria bassiana* (Balsamo) strain against *Meccus pallidipennis* (Stal)

Zumaquero Rios José Lino¹, López-Tlacomulco José Juventino¹,
Rojas García Raúl¹, Sansinenea Estibaliz²

¹Laboratorio de Parasitología y Vectores, Escuela de Biología,
Universidad Autónoma de Puebla, Puebla, México.

²Facultad de Ciencias Químicas, Universidad Autónoma de Puebla, Puebla, México.

Submitted: March 4, 2013; Approved: September 9, 2013.

Abstract

The entomopathogenic fungus *Beauveria bassiana* (Balsamo 1835) Vuillemin is an effective alternative control agent against some agricultural pests and biological vectors of important diseases such as Chagas disease. In this work we studied an isolate of *Beauveria bassiana* from the town of San Antonio Rayón, Puebla, Mexico and its entomopathogenic effects on *Meccus pallidipennis* (Stal 1872). Phylogenetic analysis using molecular comparison of the ITS and EF1 α genes, showed that the resulting cladogram places the BUAP 04 strain with a relationship closer to the AFAO 9-6 strain, within the diversity of the *B. bassiana sensu lato* group. Although there was the possibility that BUAP 04 strain was a direct descendant of strains used in campaigns of biologic control, molecular study allowed us to recognize that it was a different fungus due to numerous inserts. A strain isolated from a *T. dimiata* was evaluated for pathogenicity against another triatoma (*Meccus pallidipennis*) species obtaining an LC₅₀ of 4.16 x 10⁶ spores/mL, confirming that the BUAP 04 strain is virulent for *M. pallidipennis* and could be a good prospect for formulations to control *M. pallidipennis*.

Key words: Chagas disease, biocontrol agent, triatomines.

Introduction

Chagas disease is a major parasitic disease, caused by *Trypanosoma cruzi* and is transmitted to mammalian hosts by several species of hematophagous triatomines. Chagas disease is given little public health priority in Mexico, despite ample clinical evidence of its importance (Guzmán-Bracho, 2001). In México, at least 33 species of triatomines are known, of which 23 are unrecorded from other countries (Salazar-Schettino *et al.*, 2010; Martínez-Ibarra *et al.*, 2011). Among them, *Meccus* (former *Triatoma*) *pallidipennis* (Stal, 1872), a peridomestic vector, is considered one of the most important vectors of Chagas disease in Mexico (Martínez-Ibarra *et al.*, 2009) because it usually has high entomological indexes and it occurs in houses and chicken roosts in villages of 11 states (33%) of central, southern, eastern and western Mexico (Martínez-Ibarra *et al.*, 2009; Martínez-Ibarra *et al.*, 2011).

Biological control agents are preferred against these disease vectors. Among alternative control tools, entomopathogenic fungi are known pathogens of some triatomines (Luz and Batagin 2005; Pedrini *et al.* 2009; Safavi, 2010). However, due to the great diversity of triatomine vectors in Mexico, it is necessary to find new entomopathogenic *Beauveria* strains with lethal effects to different triatomines.

Entomopathogenic fungi are among the natural enemies of pests in agroecosystems associated with soil and are distributed around the world (Meyling *et al.*, 2012). The entomopathogenic fungus *Beauveria bassiana* (Balsamo, 1835) Vuillemin is a capable alternative control agent against some agricultural pests and biological vectors of important diseases such as Chagas disease (Luz and Batagin 2005; Pedrini *et al.* 2009; Safavi, 2010). Species recognition in *Beauveria* is difficult due to a lack of taxonomically informative morphology. A molecular phylo-

genetic analysis, based on nuclear ribosomal internal transcribed spacer (ITS) and elongation factor 1- α (EF1- α) sequences for isolates from diverse geographic origins, habitats and insect hosts, was used to resolve six well supported clades within *Beauveria bassiana* Vuillemin (Rehner and Buckley, 2005; Garrido-Jurado *et al.*, 2011).

A fungal isolate collected from a bug in a rural community in the state of Puebla, Mexico, yielded a strain that was aggressive against specimens of a controlled breeding of triatomines. Controlled development of this strain, called BUAP 04, showed morphological variations of color and colonization with respect of two reference strains AFAO 9-1 and AFAO 9-6, certified by the National Center for biological control. This fungus was aggressive in laboratory bioassays against specimens of a controlled breeding of triatomines.

Therefore in this work, we determined the morphology of this strain, phylogenetic affinities and its effect on mortality of *Meccus pallidipennis* (Stal, 1872). *M. pallidipennis* is considered one of the most important vectors of Chagas in the southern of Mexico (Martinez-Ibarra *et al.*, 2010; Martinez-Ibarra *et al.*, 2011), and is found in domestic and peridomestic habitats in the south of State of Puebla where there have been high rates of human infection with *Trypanosoma cruzi* (Zumaquero-Rios *et al.*, 2001; Sandoval-Ruiz *et al.*, 2008).

Materials and Methods

Fungus isolation and morphological identification

The fungus of this study, called BUAP 04, was isolated from a dead specimen of *Triatoma dimidiata* in San Antonio Rayón locality of Puebla State, Mexico (20°14'08 N y 97°45'00" W). This strain was grown on SDA medium (Sabouraud-Dextrose- Agar) with 0.01% of ampicillin and incubated at 28 °C. The macroscopic identification of BUAP 04 was performed using the keys of Heale *et al.* (1989). Microscopic identification of the strains was based on previously reported characterization (Goettel, 1984, 1997). The reference strains, AFAO 9-1 and AFAO 9-6, certified by the National Center for biological control, were selected because they were the strains used for biological purposes to control *Hypothenemus hampei* (coffee berry borer) and there was a need to compare the new isolate with those used in the region as agents of biological control. BUAP 04 was compared with morphological characteristics (macroscopic and microscopic) of the reference strains grown in the same conditions.

Molecular identification

BUAP 04, and reference strains, AFAO 9-1 and AFAO 9-6, were grown on PDB (Potato-dextrose-Broth) medium until the optical density reached $D.O_{600} = 1$. The cells were recovered by centrifugation and then DNA was extracted following the method described by Sherman *et al.*

(1986). The quantity of DNA was verified in an agarose gel at 0.8% by electrophoresis. Two genes were amplified by PCR (ITS and EF1- α) of the three fungal strains using a touchdown PCR procedure (Don *et al.*, 1991). The ITS (~600 bp segment) was amplified and sequenced with ITS5 (5-GGAAGTAAAAGTCGTAACAAGG-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3) primers and EF1- α (~1200 bp segment) was amplified and sequenced using EF1T (5-ATGGGTAAGGARGACAAGAC-3) and 1567R (5-ACHGTRCCRATACCACCSATCTT-3) primers using the conditions described by Rehner and Buckley (2005). PCR amplifications were performed in a total volume of 50 μ L using an Eppendorf 500W/Mastercycler thermocycler and Platinum® *Pfx* polymerase according to the instructions of the supplier (INVITROGEN). The amplified fragments were sequenced in the Centro de Investigación y Estudios Avanzados (CINVESTAV) of Irapuato. The sequence data were deposited in GenBank. The sequences of 24 taxa representing each clade basal phylogenetic analysis relevant Rehner and Buckley (2005), available in Genbank, were recovered. The sequences of *Cordyceps scarabaeicola*, which was selected as outgroup, were included. In the alignment, 600 bp of ITS and 1200 pb, which correspond to the coding region of ITS and EF1- α respectively, were used. Using the Clustal W program (Thompson *et al.*, 1997), a maximum parsimony analysis was made on the MEGA program (Tamura *et al.*, 2011). The phylogenetic analysis consisted of 500 replicas of heuristic searches for each gene. A single array with both gene sequences was then analyzed and the resulting cladograms of 500 replicas of heuristic search were obtained. A strategy, of supporting bootstrap with 1000 replicas that was recovered in the strict consensus tree, was implemented.

Evaluation on *Meccus pallidipennis*

For the virulence test, strain BUAP 04 was grown on petri dish with PDA at 28 °C during 20 days and then the spores were recovered with TWEEN 80 0.01%. The concentration of the spores was calculated by the counting in Neubauer camera and the viability of spores was determined by trypan blue exclusion method (Strober, 2001). From an initial concentration of 1×10^7 spores/mL of strain BUAP 04, seven dilutions were performed (9×10^6 , 7.5×10^6 , 7×10^6 , 4×10^6 , 2×10^6 , 1×10^6 , 5×10^5 spores/mL) and were sprayed on each group of bugs (Luz *et al.*, 2004). 1075 specimens of fifth stage of *Meccus pallidipennis* were obtained from a colony established in laboratory conditions which were sprayed with a solution of sodium hypochlorite at 0.2% to prevent a possible infection by bacteria and other fungi. The experimental design consisted of five repetitions and a control group by dilution. Each experimental unit was formed of 25 specimens within a black plastic bottle of 500 mL, where organisms were sprayed with 2 mL of the corresponding dilution and controls were sprayed with the same

volume of sterile distilled water. Once treated, the specimens were returned to strict conditions of lighting (dark containers with folded surface), photoperiod (light source exposure two hours per week), temperature (25 °C) and relative humidity (60%) (Luz *et al.*, 1998b). Mortality of the specimens was recorded 96 hours after the application of dilutions and until 25 days, that ended the experiment. Using the Probit 1.5 program (EPA, 2012) LC₅₀ and LC₉₀ were determined, and compared with previous results obtained for the strains AFAO (Zumaquero-Rios *et al.*, 2005). All organisms that died during the experiment were placed in Petri dishes with wet and sterile filter paper to highlight the emergence of the fungus. The emergent fungus (Figure 1) was reseeded in Petri dishes with PDA medium to corroborate morphological characteristics of the isolate. Using Fisher's exact test, an ANOVA was performed to compare the average mortality for the total number of repetitions of each of the eight treatments ($p = 0.05$).

Results

Morphological characteristics

The BUAP 04 and AFAO 9-6 strains showed a colony diameter average of 8 mm at eight days, a dusty appearance and cream-color, which differed in comparison to the growth diameter of 10 mm, cottony texture and lighter cream color of the strain AFAO 9-1. In the microscopic observation of BUAP 04 and AFAO 9-6 strains, short hyphae, hyaline, simple and septate were highlighted; simple conidiophores grown as irregular shape, composed of clusters of conidiogenous cells in bottle shape; sterigma in zig-zag with large amount of globose spores with a diameter of 2.4 µm approximately. In comparison, the strain AFAO 9-1 developed long hyphae, hyaline, simple and septate with

fewer conidiophores and conidiogenous cells but with the same characteristics of sterigma and the same shape and size of the spores of isolate strain.

Molecular and phylogenetic analysis

From molecular amplification of the reference (AFAO 9-1 and AFAO 9-6) and BUAP 04 strains, fragments of 600 bp of the ITS gene and 1200 bp for the EF1- α gene were obtained, which were verified by agarose gel electrophoresis using DNA molecular weight markers (1 kb plus, Invitrogen).

The amplified fragments of ITS and EF1- α genes were sequenced and were deposited in GenBank (Access numbers: JX284408 (BUAP 04), JX284409 (AFAO 9-1) and JX284410 (AFAO 9-6) for ITS sequences and JX495611 (BUAP 04), JX495612 (AFAO 9-1) and JX495613 (AFAO 9-6) for EF1- α sequences. The alignment of the ITS and EF1- α genes sequences showed high similarity (99.5 and 99 respectively) with *B. bassiana sensu lato* genes. In the alignment of the EF1- α fragment four exons (of 45, 27, 63 and 648 pb encoding a 261 aa partial protein) were found by comparison to cDNA sequences taken from 24 strains. These exons were highly conserved and that mutations of the genomic PCR fragments were mainly in the introns.

Both the ITS and EF1- α sequences of the Mexican strains remained in close phylogenetic relationship with Brazilian strains as a result of a polytomy between these strains (cladograms not shown). From phylogenetic analysis of the matrix composed of the two gene sequences, two trees of 263 steps, CI = 0.73 and RI = 79 were produced. In this analysis, BUAP 04 always formed a monophyletic



Figure 1 - *Beauveria bassiana* emerging through the coaxes and spiracles from *Meccus pallidipennis*.

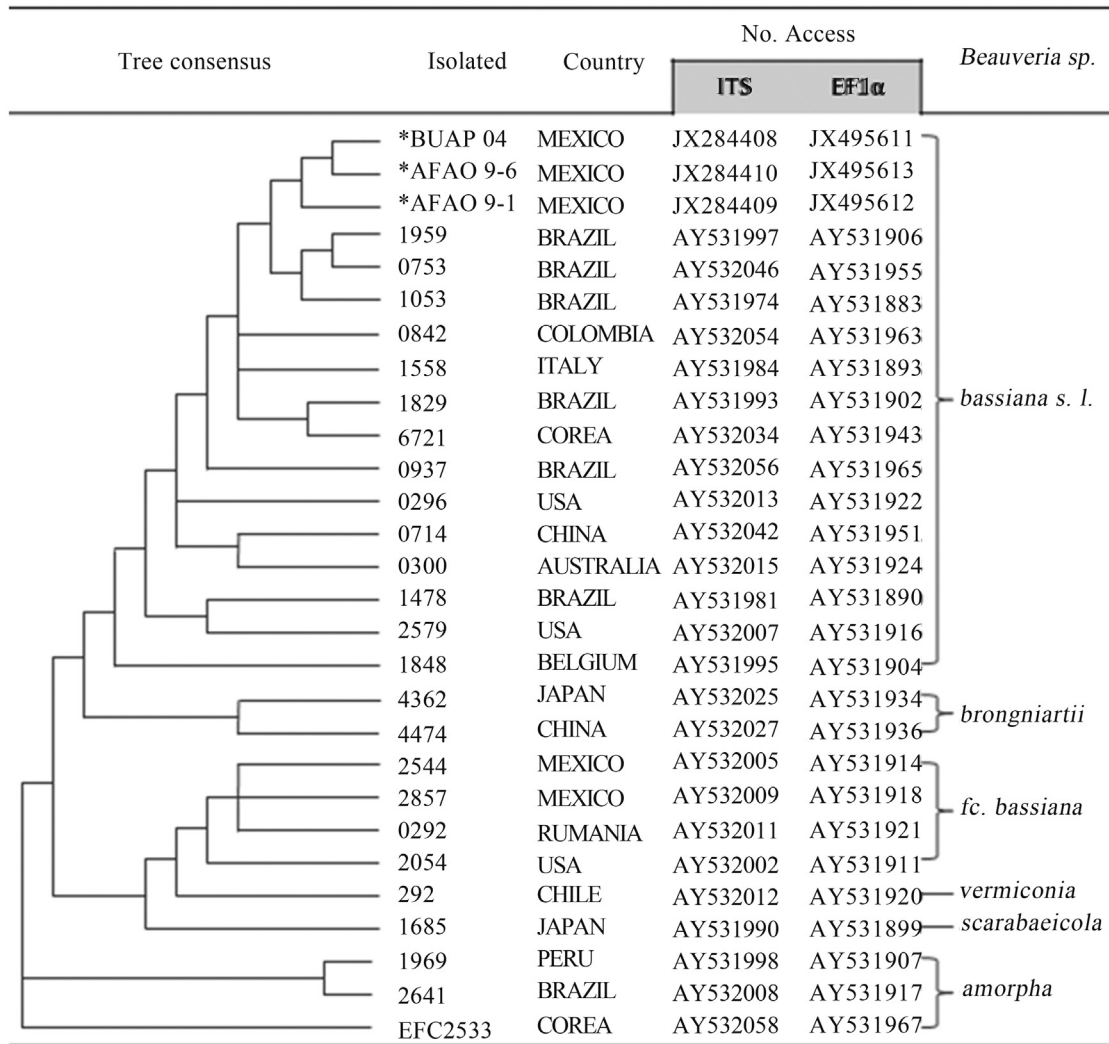


Figure 2 - Tree of strict consensus, showing the phylogenetic relationships of the isolate BUAP04 and other strains of *Beauveria* isolated from different countries. The terminal numbers correspond to sequences of reference of Rehner and Buckley, (2005). * The asterisk indicates the sequences resulted of this work.

group with AFAO reference strains. In this topology, BUAP 04 and AFAO 9-6 were nested as sister units. This group is nested in a polytomy within which we observe the monophyletic group of three Brazilian strains. The isolate and reference strains appear as one of the monophyletic derived nested group within an ancient general node, in a similar topology to that found by Rehner & Buckley (2005) that defines the group of *B. bassiana s.l. (sensu lato)*, as shown in Figure 2.

Lethal concentrations

The LC₅₀ of the BUAP 04 isolate was determined to be 4.16×10^6 spores/mL and the LC₉₀ was shown to be 1.71×10^7 spores/mL. The trypan blue staining test was negative in all cases proving that all the spores were viable. The percentage of clear mycosis in dead individuals was around 50% (Figure 3). High concentrations showed a

greater external infection (last three concentrations). Living individuals were recovered from all tested dilutions and in some specimens subjected to lower dilutions no obvious mycosis was observed. The isolate apparently caused the infertility of eggs of the surviving females of all doses.

Statistical comparison of treatments

The analysis of variance (ANOVA) showed that there are significant differences in the recorded mortality in three ranges of spore concentration (Figure 4) though it is worth mentioning that the dilution 7×10^6 spores/mL did not present significant differences from groups two and three.

Discussion

The characteristics of BUAP 04 strain identified it as *Beauveria bassiana* (Goettel, 1984, 1997; Heale et al., 1989). The morphological features of conidia are common

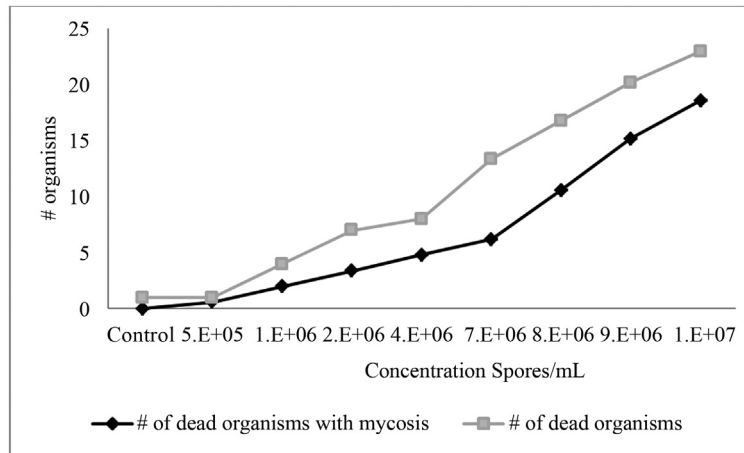


Figure 3 - Evaluation of BUAP 04 strain on *Meccus Pallidipennis*. The figure shows the number of dead organisms and dead organisms with mycosis after treatment with BUAP 04.

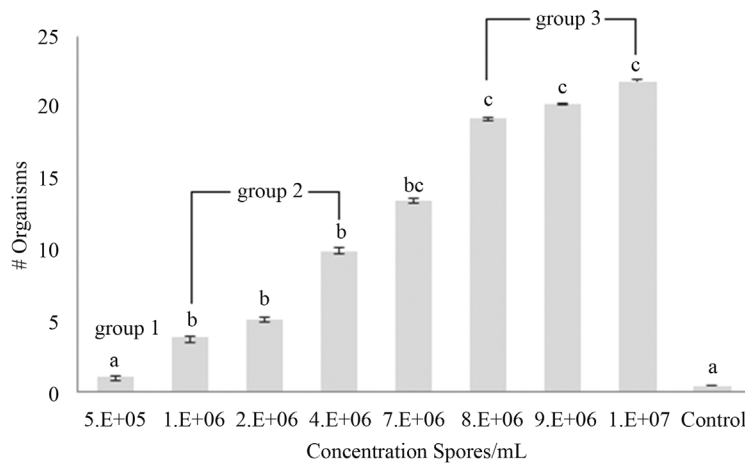


Figure 4 - Number of dead specimens of *Meccus pallidipennis* (bar = average \pm standard error) for the isolate BUAP 04 in each treatment. The same letter above the bar indicates that there is no significant difference within the group.

tools for identification in *Beauveria*. Morphological and molecular studies have shown that the broad patterns of diversity in *Beauveria* have been accurately predicted in previous morphological studies. However, they have also shown that these approaches are insufficient to investigate species such as *B. bassiana* (Rehner and Buckley, 2005). Phylogenetic analysis of the sequences of EF1-a resulted in cladograms with higher resolution, consistent with the results of Rehner and Buckley (2005) and Garrido-Jurado *et al.* (2011). The resulting topologies of phylogenetic analyses of sequences of the ITS and EF1- α genes separately, were highly similar to the topologies obtained by Rehner and Buckley (2005). Our cladogram of consensus, obtained from the analysis of both genes, matches with the characterization of *B. bassiana sensu lato* group. In this sense, the resulting cladogram allows us to identify the BUAP 04 isolate with relations closer to the AFAO 9-6 strain, within the diversity of the *B. bassiana s.l.* group. Although there was the possibility that the BUAP 04 isolate was direct de-

scendant of strains distributed throughout the campaigns of biologic control, molecular study allowed us to recognize that it was a different fungus due to the numerous inserts that are present. Information obtained from the cladogram of our work, highlights the phylogenetic proximity of the BUAP 04 isolate with strain AFAO 9-6, which shows similar morphological characteristics in the development of its cultures, as shown by the comparative morphology of the isolate.

Natural mortality in the control group corresponds to the expected since biologic cycle studies previously reported a low mortality for the fifth stage (Martinez-Ibarra *et al.*, 2009). The survivors of the group in totality were transformed into healthy imagos, which in addition to the null expression of mycosis, discards the fungal contamination of dead specimens during treatment. It was proven that BUAP 04 isolate is virulent for *Meccus pallidipennis*.

The LC₅₀ was similar to that obtained by Luz *et al.* (1998) for *Metarhizium anisopliae*, which corresponds to a

concentration of spores higher than the corresponding value for *B. bassiana* in this study. Our data were similar to those obtained by Lecuona *et al.* (2001) who recorded LC₅₀ in a range of 5.3×10^6 to 11.5×10^6 spores/mL in a period of two weeks upon infection of *T. infestans*. It can be proved that in concentrations with highest number of spores the mortality is increased in less time and the percentage of specimens that develop external mycosis is also higher. We suggest an analysis of the LC₅₀ over a longer period because, in general, few spores are required to increase mortality, to affect its ability to fertilize eggs and cause damage that suppresses feeding as was observed in the conduct of survivor specimens. The evaluation studies of the AFAO 9-1 and AFAO 9-6 strains, report a higher LC₅₀ than obtained with BUAP 04 strain, and a smaller mortality percentage at low dilutions, confirming that this strain could be a good candidate for formulations to control *M. pallidipennis* (Zumaquero-Rios *et al.*, 2005).

The observation of sporulation of the fungus in dead specimens that were treated with low concentrations, evidenced the development of massive internal hyphae that generate all known pathogenic actions on the digestive system and the reproductive tract of the insect, targets recognized in other studies where infection included ovarioles, spermatheca, and other bodies (Zumaquero and Moran unpublished data).

Acknowledgments

The authors acknowledge VIEP (Project NAT-I-97) for financial support. We want to give a special acknowledgement to Dr. Ernest Schnepf for proofreading the manuscript improving it.

References

- Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res* 19:4008.
- EPA (2012) (Environmental Pollution Agency), p. Provit, Ecological Monitoring Research, Division Environmental Monitoring Systems Laboratory. 15 edition. Cincinnati, Ohio, US.
- Garrido-Jurado I, Marquez M, Ortiz-Urquiza A, Santiago-Alvarez C, Iturriaga EA, Quesada-Moraga E, Monte E, Hermosa R (2011) Genetic analyses place most Spanish isolates of *Beauveria bassiana* in a molecular group with world-wide distribution. *BMC Microbiol* 11:84.
- Goettel MS (1984) A simple method for mass culturing entomopathogenic Hyphomycete fungi. *Microbiol. Methods* 3:15-20.
- Goettel MS (1997) Fungi: Hyphomycetes, *In*: Lacey L.A. (ed) *Manual of Techniques in insect Pathology*. E:U, pp 213-249.
- Guzmán-Bracho C (2001) Epidemiology of Chagas disease in Mexico: An update. *Trends Parasitol* 17:372-376.
- Heale JB, Isaac JE, Chandle D (1989) Prospects for strain improvement in entomopathogenic fungi. *Pesticide Science* 26:79-92.
- Lecuona RE, Edelstein JD, Berretta MF, La Rossa FR, Arcas JA (2001) Evaluation of *Beauveria bassiana* (hyphomycetes) strains as potential agents for control of *Triatoma infestans* (Hemiptera: Reduviidae). *J Med Entomol* 38:172-179.
- Luz C, Batagin I (2005) Potential of oil-based formulations of *Beauveria bassiana* to control *Triatoma infestans*. *Mycopathologia* 160:51-62.
- Luz C, Rocha LF, Nery GV, Magalhaes BP, Tigano MS (2004) Activity of oil-formulated *Beauveria bassiana* against *Triatoma sordida* in peridomestic areas in Central Brazil. *Mem. Inst Oswaldo Cruz* 99:211-218.
- Luz C, Silva IG, Cordeiro CM, Tigano MS (1998) *Beauveria bassiana* (Hyphomycetes) as a possible agent for biological control of Chagas disease vectors. *J Med Entomol* 35:977-979.
- Luz C, Tigano MS, Silva IG, Cordeiro CM, Aljanabi SM (1998) Selection of *Beauveria bassiana* and *Metarhizium anisopliae* isolates to control *Triatoma infestans*. *Mem Inst Oswaldo Cruz* 93:839-846.
- Martinez-Ibarra JA, Martinez-Hernandez F, Villalobos G, Vences-Blanco MO, Salazar-Schettino PM (2010) Update on the distribution of *Triatoma bolivari* and *Triatoma brailovskyi* (Hemiptera: Reduviidae: Triatominae) in western Mexico. *J Vector Ecol* 35:432-434.
- Martinez-Ibarra JA, Salazar-Schettino PM, Noguera-Torres B, Vences MO, Tapia-Gonzalez JM, Espinoza-Gutierrez B (2009) Occurrence of hybrids and laboratory evidence of fertility among three species of the Phyllosoma complex (Hemiptera: Reduviidae) in Mexico. *Mem Inst Oswaldo Cruz* 104:1125-1131.
- Martinez-Ibarra JA, Valencia-Navarro I, Leon-Saucedo S, Ibanez-Cervantes G, Bustos-Saldana R, Montanez-Valdez OD, Cervantes Diaz OI, Noguera-Torres B (2011) Distribution and infection of triatomines (Hemiptera, Reduviidae) by *Trypanosoma cruzi* in the state of Michoacan, Mexico. *Mem. Inst Oswaldo Cruz* 106:445-450.
- Meyling NV, Pilz C, Keller S, Widmer F, Enkerli JR (2012) Diversity of *Beauveria* spp. isolates from pollen beetles *Meligethes aeneus* in Switzerland. *J Invertebr Pathol* 109:76-82.
- Pedrini N, Mijailovsky SJ, Girotti JR, Stariolo R, Cardozo RM, Gentile A, Juarez MP (2009) Control of pyrethroid-resistant Chagas disease vectors with entomopathogenic fungi. *PLoS neglected tropical diseases* 3:e434.
- Rehner SA, Buckley E (2005) A *Beauveria* phylogeny inferred from nuclear ITS and EF1-alpha sequences: evidence for cryptic diversification and links to *Cordyceps teleomorphs*. *Mycologia* 97:84-98.
- Safavi S (2010) Isolation, Identification and Pathogenicity Assessment of a new Isolate of Entomopathogenic Fungus, *Beauveria Bassiana* in Iran. *J Plant Protect Res* 50:158-163
- Salazar-Schettino PM, Rojas-Wastavino GE, Cabrera-Bravo M, Bucio-Torres MI, Martínez Ibarra JA, Monroy-Escobar MC, Rodas Retana A, Guevara Gómez Y, Vences Blanco MO, Ruiz Hernández AL, Torres Gutiérrez E (2010) Revisión de 13 especies de la familia Triatominae (Hemiptera:

- Reduviidae) vectores de la enfermedad de Chagas en México. *J Selv And Res Soc* 1:57-80.
- Sandoval-Ruiz CA, Zumaquero-Rios JL, Rojas-Soto OR (2008) Predicting geographic and ecological distributions of triatomine species in the southern Mexican state of Puebla using ecological niche modeling. *J Med Entomol* 45:540-546.
- Sherman F, Fink GR, Hicks J (1986) *Methods in Yeast Genetics: A Laboratory Course*. Cold Spring Harbor Laboratory Press, New York.
- Strober W (2001) Trypan Blue Exclusion Test of Cell Viability, *Current Protocols in Immunology*. John Wiley & Sons, Inc.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol Biol Evol* 28:2731-2739.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25:4876-4882.
- Zumaquero-Rios JL, Valerdi-Minor G, Cid Y (2001) Triatomines en la mixteca poblana de (Xayacatlán de Bravo) Puebla. XXXVI National meeting of entomology proceedings, pp 126-131.
- Zumaquero-Rios JL, Saa-Barona O, Martínez-Ortiz P (2005) Evaluation of a bioinsecticide formulated with *Metarhizium anisopliae* and *Beauveria bassiana* for the control of vector of the Chagas disease. *Entomologia Mexicana* 4:991-994.

All the content of the journal, except where otherwise noted, is licensed under a Creative Commons License CC BY-NC.