Tropical Biomedicine 37(4): 864–870 (2020)

Isolation of *Beauveria bassiana* Pr-11 from Andean orthopterans and its effectiveness against Chagas disease vectors in Peru

Castellanos-Sánchez, P.[1], Falconi-Agapito, F.[1], Pariona, N.[3] and Paredes-Esquivel, C.[2*]
[1]Laboratory of Biological Control, Universidad Nacional Mayor de San Marcos (UNMSM), Lima, Peru
[2]Laboratory of Zoology, Department of Biology, Universitat de les Illes Balears (UIB), Palma de Mallorca, Spain
[3]Red de Estudios Moleculares Avanzados, Instituto de Ecología A.C., Veracruz, Mexico
[*]Corresponding author e-mail: claudia.paredes@uib.es
Received 15 April 2020; received in revised form 22 September 2020; accepted 24 September 2020

Abstract. Chagas disease is endemic to the Americas and is transmitted by blood-feeding kissing bugs. We evaluated the insecticidal potential of a fungus (*Beauveria bassiana* strain Pr-11) against *Triatoma infestans*, an important vector in South America. This fungal species was isolated from a locust (*Schistocerca piceifrons)* that inhabits the Central Andes region of Peru. Ten days post inoculation, this strain induced high insect mortality (97%) at low fungal concentrations (2×10^7 conidia/ml) at 70% relative humidity. The Pr-11 strain outperformed reference strain CCBLE-216 *B. bassiana*, provided by the Peruvian Ministry of Agriculture. Our results are consistent with previous reports on the virulence of this fungal strain against other insect pests. This is the first study to evaluate an orthopteran-isolated *B. bassiana* to control Chagas disease vectors. We conclude that strain *Beauveria bassiana* Pr-11 is effective against *Triatoma infestans*, resulting in a promising tool to control Chagas disease in Peru and may be used in integrated vector control programs.

INTRODUCTION

Chagas disease or American Trypanosomiasis is caused by the infection of the protozoan parasite *Trypanosoma cruzi* Chagas, 1909. It is a neglected tropical disease highly prevalent in Latin America, where between 6 to 7 million people remain infected and approximately 10 000 die each year (WHO, 2018). In Peru, it is endemic in the Northern and Southern regions of the country, where active transmission still occurs (Paredes-Esquivel *et al.*, 2010; Alroy *et al.*, 2015; Moncayo & Silveira, 2017). The disease is considered a serious problem in Peru, particularly in well-established urban communities of the southern region, following the migration of the main vector *Triatoma infestans* from rural to urban areas (Khatchikian *et al.*, 2015). In the absence of vaccines, vector control remains the main strategy to control and prevent Chagas disease. Although chemical treatments kill triatomines, there is an increasing number of reports of insecticide resistance in the region (Mougabure-Cueto & Picollo, 2015; Davila-Barboza *et al.*, 2019), indicating that there is an urgent need for new control alternatives. In Peru, pyrethroids are the most frequently used insecticides in control campaigns. However, there is limited information on the current insecticide resistance status of Chagas disease vectors in this country (Flores-Ferrer *et al.*, 2018). Yon *et al.* (2004), reported the presence of resistant *T. infestans* populations to alpha-cypermethrin, beta-cyfluthrin and deltamethrin in the southern region of the country. While the use of non-pyrethroid

insecticides such as imidacloprid and fenitrothion has been proposed as alternatives to control pyrethroid-resistant *T. infestans* populations (Carvajal *et al.*, 2014; Germano *et al.*, 2014), these have a negative impact on the environment. The use of entomopathogenic fungi represent an environmentally-friendly and cost-effective strategy, which application is simple (Pedrini *et al.*, 2009) and effective to control different stages of triatomine bugs (Flores-Villegas *et al.*, 2019). The efficacy of *Beauveria bassiana* (Bassi, 1835) as a biocontrol agent for *Triatoma infestans* (Klug, 1834) has been previously reported (Lecuona *et al.*, 2001; Luz *et al.*, 2004; Pedrini *et al.*, 2009; Forlani *et al.*, 2011), but performance depended on environmental conditions, such as humidity (Luz *et al.*, 2004). In this study, we present a highly virulent *B. bassiana* Pr-11 strain isolated from *Schistocerca piceifrons* (Lynch Arribalzaga, 1903) against *T. infestans*. Our laboratory had previously reported the high mortality induced by this strain in the same species and on pest hemipterans (Falconi *et al.*, 2010). We discuss the importance of the source of this fungal strain and its potential as biocontrol agent.

METHOD

Insect collection

Collections took place in the province of Nazca, located in the South-western region of Peru. *T. infestans* specimens were collected from domestic and peridomestic environments of triatomine infested houses, previously identified by the Ministry of Health authorities in the region. Specimens were collected using metal sticks and forceps, following specifications by Paredes-Esquivel *et al.* (2010) and transported to the insectary of the University of San Marcos (UNMSM) in Lima in metal containers covered with nylon tulle. Specimens were kept at 70% relative humidity (RH) at constant temperature (27±1°C), with light/dark cycles of 12 hours and fed on captive chickens. Triatomine eggs were collected and hatched until the F3 generation was obtained. Time since oviposition to hatching took approximately 18 days, while from N1 nymphs reached adulthood 180 days later. We selected fifth instar nymphs for bioassays they showed a lower mortality rate compared to other stages under controlled conditions.

Preparation of Fungi

The *Beauveria bassiana* PR-11 strain was isolated from adult and nymphs of *S. piceifrons*, in the locality of Huamanga (Ayacucho), located at 2761 m.a.s.l. in the central Andean region of Peru (Pariona *et al.*, 2007). This strain was named "PR" as this was the first of three insect sampling campaign (primera recolecta) and number 11 correspond to the 11th *S. piceifrons* specimen. Collected specimens were kept in the laboratory until death. These were then submerged in 5% Sodium hypoclorite per 1 minute to eliminate other micro-organisms and then washed in distilled water 3 times. Orthopterans were then kept in the humid chamber at room temperature until the appearance of fungi, which were passed to petri dishes containing Potato dextrose agar. The morphological identification was performed with an optical and electron microscope using descriptions by Barnett and Hunter (1998). This strain is characterized by forming cotton-like colonies on Potato Dextrose Agar and large conidial size (2,6 microns) (Figure 1). On the other hand, strain CCBLE-216 is a reference strain commercialized by the Ministry of Agriculture of Peru (SENASA). This was isolated from *Hypothenemus hampei* (Coleoptera: Scolitynae) from the central jungle region of Peru. After isolation, *B. bassiana* was reactivated by inoculating conidia on *T. infestans* nymphs, according to specifications by Lecuona *et al.* (2001). Conidia viability was determined by plating 5 ml of 10^{-3} conidial suspensions on 5 points PDA medium (each point is a repetition) and incubating them at 25±1°C. After 20 hours, Lacto-phenol blue solution was added to stop germination and give contrast to the

conidia. Germination was observed through direct observation using an optical microscope. Quantification of conidia was performed with the Neubauer hemocytometer.

Bioassays against triatomine nymphs

For bioassays, we obtained a 2×10^7 conidia/ml concentration of the Pr-11 strain, diluted in sterile distilled water containing 1% Tween 20. Three replicates were performed, using 10, third generation, 5th stage *T. infestans* nymphs each time. Conidial suspension was sprayed into individual nymphs for 2 seconds, approximately. Ten nymphs sprayed with Tween 20 (0.1%) were used as negative control. The *B. bassiana* CCBLE-216 strain, provided by the Peruvian Ministry of Agriculture and currently used to control crop pests, was used as a positive control. Mortality in treatments and control was recorded daily until day 16. Dead nymphs were submerged in 5% Sodium hypoclorite for 30 seconds and rinsed three times with sterile distilled water, then placed in petri dishes, covered with paper and incubated for five or six days at 20°C and 70% RH to recover the fungus.

Statistical analysis

Statistical analyses of experimental assays were performed using GraphPad Prism 8 (GraphPad). Survival after fungal infections was analyzed using Kaplan-Meier with Log-rank tests. The log-rank test (Mantelcox): is a proportional hazards regression model for comparing the Kaplan-Meier survival curves estimated for two or more groups of subjects: treated versus untreated (control) groups. This renders chi-square test and P value to determine if the survival curves are significantly different. We compared significant differences among the three replicates and differences between each replicate vs. the negative and positive controls. Statistical significance was assessed at $P<0.05$. The LT50 and LT80 values the median lethal time to cause the mortality of 50% or 80% of insects, respectively, were determined for each replicate using the logistic regression analysis method.

RESULTS

Dead *T. infestans* insects showed fungal hyphae appearing in the antennal, head and thorax regions. Colonies of the Pr-11 strain exhibited flat white cotton-like appearance, while those from the CCBLE-216 strain developed some yellowish coloration and sno appearance. The morphology of the conidial structures and synnema was typical of this species. The average conidial size of the Pr-11 strain was 2,6 microns (Figure 1). Mortality with the *B. bassiana* Pr-11 strain was first recorded at day 5, with a steady increase on the following days. The cumulative media mortality for the three replicates reached 94% in the treated *T. infestans* insects, 10 days post inoculation (dpi). In contrast, the cumulative mortality 10 days after the application of the *B. bassiana* reference strain (CCBLE-216) was 62%. The average lethal times (LT50 and LT80) of the Pr-11 strain were 6.35 and 7.60 days, respectively, while these were 8.61 and 11.2 for the CCBLE-216 strain. No mortality was registered in the negative control group. After death *B. bassiana* was isolated from all exposed insects to confirm that death was the result of an infection by this species. No significant differences in the survival rates after exposure were observed among the three replicates (Figure 2); but survival curves differed significantly when comparing treated and negative control groups (0.01%) (log-rank Mantel-Cox test: Pr-11 Rep1, χ^2: 26.51, $P<0.0001$; Pr-11 Rep2, χ^2: 26.66, $P<0.0001$ and Pr-11 Rep3, χ^2: 25.98, $P<0.0001$). Significant differences were also observed when comparing each replicate of the Pr-11 treatment with the positive control group (log-rank Mantel-Cox test: Pr-11 Rep1, χ^2: 9.023, $P<0.01$; Pr-11 Rep2, χ^2: 9.806, $P<0.01$ and Pr-11 Rep3, χ^2: 7.645, $P<0.01$) (Figure 2). The Pr-11 strain showed a higher virulence than the reference strain, which was reflected by the hazard ratio (Mantel-Haenszel ratio > 2.0). These results indicate that the rate of death with the Pr-11 strain was twice the

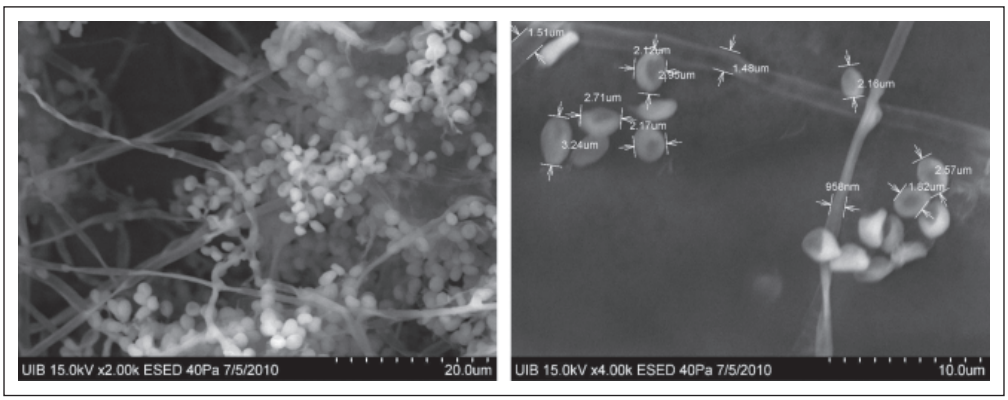

Figure 1. Electron microphotograph of *Beauveria bassiana* Pr-11 showing dense clusters of globose conidiogenous cells showing a zig-zag appearance (left). Average conidial size was 2.6 microns (right).

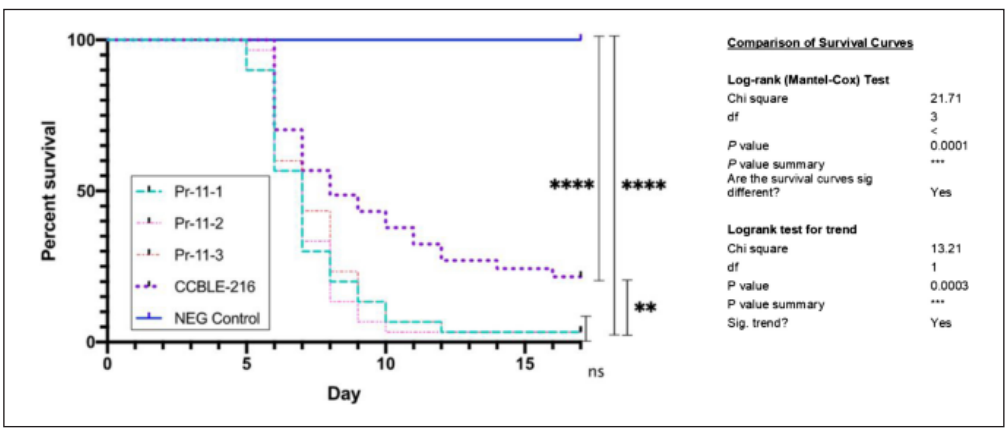

Figure 2. Survival of *Triatoma infestans* after application of *Beauveria bassiana* Pr-11 strain. **Legend:** Kaplan-Meier plots for the survival of the triatomines from the three treatments are shown. *B. bassiana* Pr-11 was evaluated by triplicate: Pr-11-1 (replicate 1), Pr-11-2 (replicate 2) and Pr-11-3 (replicate 3). CLBE strain was used as a positive control. Triatomines used as negative control are represented by solid lines; triatomines exposed to *B. bassiana* are represented by dashed lines. Statistical significance is represented with asterisks (**P<0.01; ****P<0.0001; ns: no statistical significant difference).

Table 1. Survival proportions calculated using the Kaplan Meier method. The three replicates of strain Pr-11 is shown in parenthesis. Number represent percentage of individuals alive per day

Day	Pr-11 (1)	Pr-11 (2)	Pr-11 (3)	CCBLE-216	Neg control
0	100.00	100.00	100.00	100.00	100.00
5	90.00	96.67	90.00		
6	56.67	56.67	60.00	70.27	
7	30.00	33.33	43.33	56.76	
8	20.00	13.33	23.33	48.65	
9	13.33	6.67	13.33	43.24	
10	6.67	3.33	6.67	37.84	
11				32.43	
12	3.33		3.33	27.03	
14				24.32	
16				21.62	
17	3.33	3.33	3.33	21.62	100.00

rate when compared with the reference strain CCBLE-216.

DISCUSSION

This study shows that the *B. bassiana* Pr-11 strain was highly virulent against fifth star nymphs of *T. infestans* under laboratory conditions. This strain has been found as a result of a long-term search for entomopathogenic fungi from different insect hosts, conducted by our team since 2003. At the same environmental conditions, we have previously showed that a 10^8 concentration of the Pr-11 strain was effective against the cotton pest *Dysdercus peruvianus* (Hemiptera:Pyrrhocoridae), reporting a LT50=5,6 days and 19,2 days and outperforming other 50 fungal isolates (Falconi *et al.*, 2010). Similarly, Pariona *et al.* (2007) showed promising results of this strain against *S. piceifrons*, at a 10^8 conidial concentration, resulting in a LT50=8.34 and LT80=9.6 days. These results indicate that *B. bassiana* may be more pathogenic against hosts other than the original one.

Conidial concentration and relative humidity (RH) are crucial factors that may affect the performance *B. bassiana*. Luz *et al.* (2004) reported two strains (10^8 conidia/ml) that induced a 100% mortality in *T. infestans* at a RH > 98%, but mortality was reduced dramatically at RH=75%. The Pr-11 strain induced 97% mortality in the treated triatomines at a lower concentration (2×10^7 conidia/ml and humidity (RH=70%). Our results also outperformed those from Lecuona *et al.* (2001) who needed more days to obtain similar results and a higher fungal concentration (>10^8 conidia/ml). Vazquez-Martinez *et al.* (2014) also reported a 90% mortality rate at a RH=75%, but they occurred 19 dpi, while the Pr-11 strain caused a 97% mortality 10 dpi (Figure 2). High mortality rates were obtained by Pedrini *et al.* (2009), but using a 10^9 conidia/ml concentration of the GHA strain (Laverlam International, Butte, USA).

Strong variations in the virulence of *B. bassiana* strains have been observed (Luz *et al.*, 2004) and these have been associated to several aspects of the host-pathogen interaction (Rohrlich *et al.*, 2018). The mode of action of this pathogen consists on a first adhesion step to the insect cuticle and later hyphal penetration and proliferation of the cuticle of the infected host (Gabarty *et al.*, 2014). However, virulence factors such as the amount of chitinases produced (Lovera *et al.*, 2020) may explain the variations found in different strains. It is generally assumed that fungal pathogens are somehow specific to their insect host (Romaña & Fargues, 1987). However, host specificity most likely do not play a role in *B. bassiana* pathogeneicity (Lecuona *et al.*, 2001; Bidochka *et al.*, 2002). Here we report a *B. bassiana* strain isolated from Orthopterans with promising potential as biocontrol agents of triatomines. The performance of *B. bassiana* seem to be affected from environmental conditions, with those strains isolated from cold regions growing better at lower temperatures (Bidochka *et al.*, 2002). The strain we have used in the bioassays is native from the Central Andes region of Peru, characterized by dramatic variations in temperatures during the day. Whether or not this may represent an advantage in field applications is something that needs further studies. The performance of this strain can be further improved by adding synthetic insect-like hydrocarbon sources into the media culture as it has been reported to increase *B. bassiana* virulence (Pedrini *et al.*, 2009), or by adding chitinases that favour the invasion process (Lovera *et al.*, 2020). The use of attraction infectious traps protects conidia from UV light, increasing its residual effect (Forlani *et al.*, 2011).

Although there are still many open questions regarding the future of fungal bioinsecticides, they are promising tools, particularly in areas where insecticide resistance is increasing.

Acknowledgements. Authors would like to thank the financial support given by the Ministry of Health of Peru (USAID-MINSA fund) and Ms. Hilda Gómez from the Ministry of Agriculture of Peru for providing reference strain.

Conflict of Interests

The authors declare that they have no conflict of interests.

REFERENCES

Alroy, K.A., Huang, C., Gilman, R.H., Quispe-Machaca, V.R., Marks, M.A., Ancca-Juarez, J., Hillyard, M., Verastegui, M., Sanchez, G., Cabrera, L., Vidal, E., Billig, E.M.W., Cama, V.A., Naquira, C., Bern, C., Levy, M.Z. & Working Grp Chagas Dis, P. (2015). Prevalence and transmission of *Trypanosoma cruzi* in people of rural communities of the high jungle of Northern Peru. *Plos Neglected Tropical Diseases* **9**(5): e0003779.

Barnett, H.L. & Hunter, B. (1998). *Ilustrate Genera of Imperfec Fungi*, St Paul, Minnesota (USA).

Bidochka, M.J., Menzies, F.V. & Kamp, A.M. (2002). Genetic groups of the insect-pathogenic fungus *Beauveria bassiana* are associated with habitat and thermal growth preferences. *Archives of Microbiology* **178**: 531-537.

Carvajal, G., Picollo, M.I. & Toloza, A.C. (2014). Is imidacloprid an effective alternative for controlling pyrethroid-resistant populations of *Triatoma infestans* (Hemiptera: Reduviidae) in the Gran Chaco ecoregion? *Memorias Do Instituto Oswaldo Cruz* **109**: 761-766.

Davila-Barboza, J., Villanueva-Segura, O.K., Lopez-Monroy, B., Ponce-Garcia, G., Bobadilla-Utrera, C., Montes-Rincon, M., Molina-Garza, Z.J., Arredondo-Jimenez, J.I., Rodriguez-Sanchez, I.P., Manrique-Saide, P.C. & Flores, A.E. (2019). Novel Kdr mutations (K964R and A943V) in pyrethroid-resistant populations of Triatoma mazzottii and Triatoma longipennis from Mexico and detoxifying enzymes. *Insect Science*, **26**: 809-820.

Falconi, F., Flores, A. & Castellanos, P. (2010). Letalidad de hongos entomopatogenos sobre *Dysdercus peruvianus* (Hemiptera: Pirrhocoridae). *Revista Peruana de Biología* **17**: 225-229.

Flores-Ferrer, A., Marcou, O., Waleckx, E., Dumonteil, E. & Gourbiere, S. (2018). Evolutionary ecology of Chagas disease; what do we know and what do we need? *Evolutionary Applications* **11**: 470-487.

Flores-Villegas, A.L., Cabrera-Bravo, M., Perez-Torres, A., Cordoba-Aguilar, A., Salazar-Schettino, P.M., Hernandez-Velazquez, V.M. & Toriello, C. (2019). Effects on *Meccus pallidipennis* (Hemiptera: Reduviidae) eggs exposed to entomopathogenic fungi: exploring alternatives to control Chagas disease. *Journal of Medical Entomology* **56**: 284-290.

Forlani, L., Pedrini, N. & Juarez, M.P. (2011). Contribution of the horizontal transmission of the entomopathogenic fungus *Beauveria bassiana* to the overall performance of a fungal powder formulation against *Triatoma infestans*. *Research and Reports in Tropical Medicine* **2**: 135-140.

Gabarty, A., Salem, H.M., Fouda, M.A., Abas, A.A. & Ibrahim, A.A. (2014). Pathogencity induced by the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in *Agrotis ipsilon* (Hufn.). *Journal of Radiation Research and Applied Science* **7**: 95-100.

Germano, M., Picollo, M.I., Spillmann, C. & Mougabure-Cueto, G. (2014). Fenitrothion: an alternative insecticide for the control of deltamethrin-resistant populations of *Triatoma infestans* in northern Argentina. *Medical and Veterinary Entomology* **28**: 21-25.

Khatchikian, C.E., Foley, E.A., Barbu, C.M., Hwang, J., Ancca-Juarez, J., Borrini-Mayori, K., Quispe-Machaca, V.R., Naquira, C., Brisson, D., Levy, M.Z. & Chagas Dis Working Grp, A. (2015). Population structure of the Chagas disease vector *Triatoma infestans* in an urban environment. *Plos Neglected Tropical Diseases* **9**.

Lecuona, R.E., Edelstein, J.D., Berretta, M.F., La Rossa, F.R. & Arcas, J.A. (2001). Evaluation of *Beauveria bassiana* (Hyphomycetes) strains as potential agents for control of *Triatoma infestans* (Hemiptera: Reduviidae). *Journal of Medical Entomology* **38**: 172-179.

Lovera, A., Belaich, M., Villamizar, L., Patarroyo, M.A. & Barrera, G. (2020). Enhanced virulence of *Beauveria bassiana* against *Diatraea saccharalis* using a soluble recombinant enzyme with endo- and exochitinase activity. *Biological Control* **144**.

Luz, C., Rocha, L.F.N. & Nery, G.V. (2004). Detection of entomopathogenic fungi in peridomestic triatomine-infested areas in Central Brazil and fungal activity against *Triatoma infestans* (Klug) (Herniptera: Reduviidae). *Neotropical Entomology* **33**: 783-791.

Moncayo, A. & Silveira, A.C. (2017). *American Trypanosomiasis Chagas Disease. One Hundred Years of Research*. Elsevier. pp. 59-88.

Mougabure-Cueto, G. & Picollo, M.I. (2015). Insecticide resistance in vector Chagas disease: Evolution, mechanisms and management. *Acta Tropica* **149**: 70-85.

Paredes-Esquivel, C., Lecaros, E., Aguliar-Rosales, M., Acosta, H.S. & Castellanos, P. (2010). Entomological Factors Affecting the Low Endemicity of Chagas Disease in Nazca, Southwestern Peru. *Vector-Borne and Zoonotic Diseases* **10**: 341-346.

Pariona, N., Castellanos, P. & León, E. (2007). Capacidad entomocida de cepas nativas de *Beauveria* sp. sobre *Schistocerca piceifrons peruviana* (Lynch Arribalzaga, 1903). *Revista Peruana de Biología* **14**: 253-257.

Pedrini, N., Mijailovsky, S.J., Girotti, J.R., Stariolo, R., Cardozo, R.M., Gentile, A. & Juarez, M.P. (2009). Control of pyrethroid-resistant Chagas disease vectors with entomopathogenic fungi. *Plos Neglected Tropical Diseases* **3**(5): e434.

Rohrlich, C., Merle, I., Hassani, I.M., Verger, M., Zuin, M., Besse, S., Robene, I., Nibouche, S. & Costet, L. (2018). Variation in physiological host range in three strains of two species of the entomopathogenic fungus *Beauveria*. *Plos One* **13**(7): e0199199.

Romaña, C. & Fargues, J. (1987). Sensibilité des larves de l'hémiptère hémathophage, *Rhodnius prolixus* [Triatominae] aux hyphomycètes entomopathogènes. *Entomophaga* **32**: 167-179.

Vazquez-Martinez, M.G., Cirerol-Cruz, B.E., Torres-Estrada, J.L. & Lopez, M.H.R. (2014). Potential for entomopathogenic fungi to control *Triatoma dimidiata* (Hemiptera: Reduviidae), a vector of Chagas disease in Mexico. *Revista Da Sociedade Brasileira De Medicina Tropical* **47**: 716-722.

WHO. (2018). Chagas disease (American trypanosomiasis) (accessed on 25 January 2020). World Health Organization, Geneva.

Yon, C., Balta, R., García, N., Troyes, M., Cumpa, H. & Valdivia, A. (2004). Susceptibilidad y resistencia de *Triatoma infestans* y *Panstrongylus herreri* a los insecticidas piretroides. Perú. 2001. *Revista Peruana de Medicina Experimental y Salud Publica* **21**(3).