Short Communication

Virulence of *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) Strains against the Eggs and Larvae of *Rhipicephalus sanguineus* (Acari: Ixodidae)

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A B S T R A C T

The aim of this research was to evaluate the virulence of *Metarhizium anisopliae* on the eggs and larvae of *Rhipicephalus sanguineus*, as well as the LC_{50} on *R. sanguineus* larvae, were determined and the germination (at 18 and 24 h post-inoculation) and radial growth (after 14 days) of four strains of *M. anisopliae*. We evaluated the pathogenicity on the eggs and larvae of all tested strains of *R. sanguineus* at day 3 and 7. The LC_{50} of four strains of *M. anisopliae* was determined by Probit analysis, and a chi-square test was used to determine the relationship of LC_{50} with variable radial growth and germination at 18 and 24 h. Germination at 18 h and 24 h ranged from 83.8–92.5% and 89.5–100%, respectively. The average radial growth ranged from 2.3–3.3 mm per day. The pathogenicity results for eggs and larvae showed 100% mortality for both biological stages. The Ma4 strain showed an outstanding LC_{50} value with $1.78 \times 10E6$ conidia mL^{-1}. *M. anisopliae* is effective for controlling *R. sanguineus*, and that the radial growth characteristics and germination were not correlated with the fungal virulence against *R. sanguineus* larvae. We can conclude that there are differences between the evaluated strains of *M. anisopliae* in regard to germination at 18 and 24 h, radial growth and virulence against the larvae of *R. sanguineus*. There is also no relationship between the biological characteristics and virulence of *M. anisopliae* against *R. microplus* larvae.

R *hipicephalus sanguineus* (Latreille) *sensu stricto* (Acari: Ixodidae) is a hematophagous ectoparasite. Its main host is the domestic dog, but it can also parasitize other domestic and wild animals as well as humans, and may also act as a vector-transmitter of various pathogens (Dantas-Torres, 2010).

In order to control this tick, different chemical acaricides are used; however, the inappropriate use of these products adversely impacts human and animal health, contributes to environmental pollution and drives the development of resistance in ticks (Encinosa-Guzmán *et al.*, 2016). Therefore, sustainable alternative methods that are environmentally safer and profitable are sought after (Dantas-Torres, 2008).

Entomopathogenic fungi (EPF) such as *Metarhizium anisopliae sensu lato* (Metschnickoff) Sorokin and *Beauveria bassiana sensu lato* (Balsamo) Vuillemin have demonstrated ability to kill the larvae of *R. sanguineus*

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under laboratory conditions, with mortalities ranging from 70–100% of larvae and up to 90% of eggs (Prette *et al.*, 2005).

Investigation of the efficacy of EPF on *R. sanguineus* has facilitated the selection of outstanding strains from different geographical regions; however, the lethal concentrations of the tested strains have not yet been reported. Consequently, it is necessary to determine the lethal concentrations (LC$_{50}$) of strains of EPF *M. anisopliae* to differentiate and subsequently use the most virulent strain under controlled conditions for animal or plant strains. Together with this data, determining the growth characteristics of each strain under laboratory conditions is useful for selecting candidates for use in *in vivo* models.

This paper summarizes the pathogenicity of four strains of *M. anisopliae sensu lato*, all native to the state of Guanajuato, Mexico, against the eggs and unfed larvae of *R. sanguineus sensu stricto*. We also evaluated the characteristics of spore germination of tested strains.

Materials and methods

R. sanguineus sensu stricto engorged ticks were collected from domestic dogs in the city of Irapuato in the state of Guanajuato, Mexico. Specimens were collected manually and placed in a Petri dish for transportation to the laboratory. The ticks were placed in individual Petri dishes until oviposition and maintained at 25°C and 85% relative humidity (RH). The eggs were incubated at 28°C and 85% RH until the larvae hatched. We waited until the larvae were 7 days post-hatching to use them in the bioassay. Another part of the ovigerous mass was placed in a Petri dish for 3 days before being used in the bioassay (Angel-Sahagún *et al.*, 2010).

The fungal strains used in this study were obtained from the Mycological Collection of the Laboratory of Parasitology and Biological Control of the University of Guanajuato (Table I). Four strains of *M. anisopliae* (Ma3, Ma4, Ma7 and Ma8) were grown in Sabouraud dextrose agar supplemented with 1% yeast extract and 500 ppm

of chloramphenicol for 21 days at 25±1°C under a 12-h light/dark cycle (Angel-Sahagún *et al.*, 2010). Conidia were harvested by scraping the culture medium, and then resuspended in sterile distilled water with 0.1% (v/v) Tween 80. Conidia were diluted to obtain the concentrations used in the different evaluations (Angel-Sahagún *et al.*, 2010).

For each strain of *M. anisopliae*, a suspension of 1×10^6 conidia mL^{-1} was adjusted with 0.1% Tween 80 (v/v), then 100 μL was inoculated in Petri dishes with Sabouraud dextrose agar. The inoculum was distributed with a cell disperser and the germination (G) was assessed after 18 and 24 h. Each count consisted of 100 conidia with four replicates per treatment. A conidium was considered to have germinated only if a germ tube was at least as long as the length of an ungerminated conidium.

For radial growth (RG) initially, Petri dishes containing Sabouraud dextrose agar were inoculated with 100μL of the conidia suspension at a concentration of 1×10^6 conidia mL^{-1}. The Petri dishes were incubated at 25±1°C for between 3 and 8 days post-inoculation (PI). Once uniform growth was observed on Sabouraud dextrose agar in the Petri dish, a mycelial unsporulated disc of 6 cm in diameter was extracted using a punch and placed individually in the center of another dish and incubated at 25±1°C (Dimbi *et al.*, 2004). RG was recorded until day 14 PI from the center of the colony in four equidistant radii previously marked on each Petri dish (Fargues *et al.*, 1999).

For pathogenicity tests on the eggs and larvae of *R. sanguineus*, conidia concentrations of 1×10^7 and 1×10^8 conidia mL^{-1}, respectively, were used. For virulence bioassays, seven concentrations of conidia (1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia mL^{-1}) were prepared. In all treatments, the experimental unit consisted of 25 specimens.

The evaluation of pathogenicity against eggs was performed according to Gindin *et al.* (2009). A total of four strains of EPF of *M. anisopliae* were tested, with four replications per treatment. The inoculation was performed

Table I.- Germination, radial growth, fungal infection and LC$_{50}$ (95% confidence interval) of entomopathogenic fungi against the eggs and larvae of *Rhipicephalus sanguineus* under laboratory conditions.

Strain	Place of isolation	Germination (%)		Radial growth (mm day^{-1})	Mycosis (%)		Virulence, LC$_{50}$, (IC) conidia mL^{-1}
		18 h	24 h		Eggs	Larvae	
Ma3	Abasolo, Guanajuato	83.8 (2.9)^c	89.5 (6.0)^b	3.3 (0.1)^a	100.0 (0.0)^a	100.0 (0.0)^a	2.35×10^6 (1.06×10^6-6.57×10^6)
Ma4	Guanajuato, Guanajuato	86.8 (4.7)^{bc}	98.0 (0.8)^a	2.3 (0.3)^c	100.0 (0.0)^a	100.0 (0.0)^a	1.78×10^6 (4.59×10^5-6.68×10^6)
Ma7	Silao, Guanajuato	92.5 (3.6)^a	100.0 (0.0)^a	2.7 (0.2)^b	100.0 (0.0)^a	100.0 (0.0)^a	2.88×10^7 (1.25×10^7-6.30×10^8)
Ma8	Irapuato, Guanajuato	90.8 (1.5)^{ab}	98.5 (1.2)^a	2.5(0.1)^{bc}	100.0 (0.0)^a	100.0 (0.0)^a	5.49×10^8 (1.64×10^7-1.82×10^{10})
Witness		0.0 (0.0)^d	0.0 (0.0)^c	0.0 (0.0)^d	0.0 (0.0)^b	0.0 (0.0)^b	-
P-value		0.2133	0.2183	0.2231	<0.0001	<0.0001	-

Means (± standard deviation) with different letters within the same column are significantly different (P ≤ 0.05).

by applying 500μL of a conidia suspension of 1×10^7 conidia mL^{-1} to each group of eggs. The control group was inoculated with sterile distilled water containing 0.1% Tween 80. The dishes were incubated at 25±1°C under a 12-h light/dark photoperiod. For each treatment, the number of eggs infested with fungi was observed and recorded every 48 h until the emergence of larvae in the control.

For evaluation of pathogenicity against larvae, groups of 25 larvae were stuck onto 5-cm thick pieces of adhesive tape, then immersed for 5 s in an suspension of conidia at 1×10^8 mL^{-1} (Kaaya et al., 1996). The control consisted of submerging the larvae in a solution containing Tween 80 (0.1%). Bioassays were incubated at 25±1°C under a 12-h light/dark photoperiod. Larval mortality was recorded every 48 h for 16 days (Angel-Sahagún et al., 2010).

Virulence assays were developed using seven concentrations of conidia ranging from 1×10^2 to 1×10^8 conidia mL^{-1} (Angel-Sahagún et al., 2010). Groups of 25 R. sanguineus larvae were used for each treatment, each of which had four replicates. Larvae were placed on a 5-cm thick piece of adhesive tape and immersed in the corresponding concentration of conidia for 5 s, then placed in a Petri dish containing a double layer of paper moistened with sterile distilled water. The larval control treatment involved immersion in a solution of Tween 80 (0.1%). The dishes were incubated at 25 ± 1°C under a 12-h light/dark cycle. The number of infected larvae was recorded every 48 h and fungal infection was confirmed upon fungal sporulation under larvae body (Smith et al., 2000).

For statistical analysis, the germination (G) percentage values were transformed to Arc Sen√x/100. An ANOVA with a completely randomized design was performed, followed by between-group comparison by Tukey´s test (P = 0.05) for the following variables used to assess pathogenicity: G, RG (cumulative growth after 14 days), and the percentage of mycosis eggs and larvae. All analyses were performed using SAS software (SAS, 1997). A Probit analysis was used to assess the results of the virulence bioassay in order to determine the lethal concentrations 50% of the population (Angel-Sahagún et al., 2010). In addition, a chi-square (X^2) test (95% confidence) was performed to investigate correlations between growth variables (G, RG) and virulence (LC$_{50}$) of the tested strains.

Results and discussion

The viability of conidia at 18 and 24 h (PI) ranged from 83.8–92.5% and from 89.5–100%, respectively. The outstanding strain at both 18 and 24 h PI was Ma7. The average RG per day varied from 2.5–3.9 mm, and the outstanding strains were Ma7 and Ma8 (Table I). The ANOVA of germination at 18 and 24 h and radial growth showed statistically significant differences (P<0.05), and the Tukey (P<0.05) post hoc test formed three groups (Table I).

The results in our study corroborate that there are differences in the percentage of germination, radial growth and LC$_{50}$ between the strains tested. At 24 h, we observed 100% germination of conidia that had been incubated at 25°C. A value of 94.9% germination was previously reported by Dimbi et al. (2004) for strains of M. anisopliae isolated mainly from the soil, and these authors suggested that the geographical origin of the strains is probably related to germination characteristics.

Magalhâes et al. (2003) observed differences in radial growth between strains of M. anisopliae at 14 days, with values reported to range between 2.52 and 4.46 mm day^{-1}, which are similar to those reported for the Ma3, Ma7, and Ma8 strains in our study. Furthermore, Dimbi et al. (2004) reported a radial growth of M. anisopliae between 2.0 and 2.27 mm day^{-1}, which is less than the values obtained in the present study. This could be attributed to the germination characteristics of the strains at different geographical origins, which may influence the biological characteristics.

The four strains tested were found to have pathogenicity against the eggs and larvae of R. sanguineus, with 100% infection observed at 1×10^8 and 1×10^7 conidia mL^{-1}, respectively. The mycosis was observed from the sixth day post inoculation. The ANOVA showed statistically significant differences between EPF and the control (P<0.0001) (Table I).

The LC$_{50}$ values estimated for the four strains of M. anisopliae ranged from 1.78×10^6 to 5.49×10^8 conidia mL^{-1}. The Ma4 strain was the most outstanding and Ma8 was the least outstanding, with an LC$_{50}$ of 5.49×10^8 conidia/mL^{-1}.

Our results related to the pathogenicity against tick eggs of R. sanguineus are in agreement with those reported by Gindin et al. (2002). These authors have evaluated M. anisopliae strains on eggs of different tick species under laboratory conditions, and reported mortalities up to 98%. They also found differences in mortality between strains of the same species. Other reports focused on M. anisopliae have found similar efficacy between the strains evaluated in relation to tick species and the biological egg stage, as the greatest susceptibility was noted during the development phase.

To our knowledge, no studies in the literature have reported the 50% lethal concentrations (LC$_{50}$) of strains of EPF on the larvae of R. sanguineus. In the present study, we found that the more virulent strains of fungi had higher and lower LC$_{50}$ values against R. sanguineus than those reported for other ticks in a study by Angel-Sahagún et al.

(2010), who reported LC_{50} values ranging from 1.1×10^4 and 9.2×10^4 conidia mL^{-1} for strains of *M. anisopliae* on larvae of *R. micoplus*. This is not conclusive, as Quinelato *et al.* (2012) determined an LC_{50} of 1.14×10^7 conidia mL^{-1} for *M. anisopliae* on *R. microplus*. These LC_{50} results are different to those reported in this study; however, they were conducted using a different species of tick. These differences in results suggest that *M. anisopliae* has varying virulence between members of the same species, which may be due to strain characteristics such as the production of toxins, biological stage of the host and the environmental conditions. The results of the chi-square (X^2) test indicate that there is no association between germination at 18 h PI (P>0.2133), 24 h PI (P>0.2183) or radial growth (P>0.2231) with the LC_{50} results of the four strains tested in this study.

In the present study, we failed to demonstrate an association between germination and radial growth on the virulence of *M. anisopliae* against the larvae of *R. sanguineus*. The EPF *M. anisopliae* can germinate and have a radial growth non-efficiently and have an outstanding LC_{50}. Maniania and Fargues (1992) noted that the radial growth of *Isaria fumosorosea* (= *Paecilomyces fumosoroseus*) (Wize) A. H. S. Br. and G. Sm. is related to infection of *Spodoptera littoralis* Boisduval. Furthermore, Inglis *et al.* (1999) assessed the effect of *M. flavoviride* (Gams and Rozsypal) on *Melanoplus sanguinipes* (Fabricius) and found that germination was correlated with pathogenicity. This is likely to be due to differences between the species of fungus and/or pest species, and even probably due to differences between agricultural and livestock pests, particularly considering that the latter part of their life cycle is dependent on the host.

Conclusions

We can conclude that there are differences between the evaluated strains of *M. anisopliae* in regard to germination at 18 and 24 h, radial growth and virulence against the larvae of *R. sanguineus*. There is also no relationship between the biological characteristics and virulence of *M. anisopliae* against *R. microplus* larvae.

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Statement of conflict of interest

The authors declare that they have no conflict of interest.

References

Angel-Sahagún, C.A., Lezama-Gutiérrez, R., Molina-Ochoa, J., Pescador-Rubio, A., Skoda, S.R., Cruz-Vázquez, C., Lorenzoni, A.G., Galindo-Velasco, E., Fragoso-Sánchez, E.H. and Foster J.E., 2010. *Vet. Parasitol.,* **170**: 278-286. https://doi.org/10.1016/j.vetpar.2010.02.037

Dantas-Torres, F., 2008. *Vet. Parasitol.,* **152**: 173–185. https://doi.org/10.1016/j.vetpar.2007.12.030

Dantas-Torres, F., 2010. *Parasites Vectors,* **3**: 26. https://doi.org/10.1186/1756-3305-3-26

Dimbi, S., Maniania, N.K., Lux, S.A. and Mueke, J.M., 2004. *Biocontrol,* **49**: 83–94. https://doi.org/10.1023/B:BICO.0000009397.84153.79

Encinosa-Guzmán, P.E., Bello-Soto, Y. and Rodríguez-Mallon, A., 2016. *Int. J. Acarol.,* **42**: 18–25. https://doi.org/10.1080/01647954.2015.1113309

Fargues, J., Goettel, S., Smits, N., Ouedraogo, A. and Rougier, M., 1999. *Mycology,* **89**: 383–392. https://doi.org/10.1080/00275514.1997.12026797

Gindin, G., Samish, M., Zangi, G., Mishoutchenko, A. and Glanzer, I., 2002. *Exp. appl. Acarol.,* **28**: 283–288. https://doi.org/10.1023/A:1025379307255

Gindin, G., Ment, D., Rot, A., Glazer, I. and Samish, M., 2009. *J. med. Ent.* **46**: 533–538. https://doi.org/10.1603/033.046.0318

Inglis, G.D., Duke, G.M., Kawchuk, L.M. and Goettel, M.S., 1999. *Biol. Contr.,* **14**: 111–120. https://doi.org/10.1006/bcon.1998.0666

Kaaya, G.P., Mwangi, E.N. and Ouna, E.A., 1996. *J. Inverteb. Pathol.,* **67**: 15–20. https://doi.org/10.1006/jipa.1996.0003

Magalhâes, B.P., Silvana-Tigano, M., Martins, I., Frazâo, H. and Ramirez, H.G., 2003. *Pesqui. Agropecu. Bras.,* **38**: 1469–1475. https://doi.org/10.1590/S0100-204X2003001200014

Maniania, N.K. and Fargues, J., 1992. *J. appl. Ent.,* **113**: 518–524. https://doi.org/10.1111/j.1439-0418.1992.tb00698.x

Prette, N., Monteiro, A.C., Garcia, M.V. and Soares, V.E., 2005. *Ciênc. Rural,* **35**: 855–861. https://doi.org/10.1590/S0103-84782005000400017

Smith, K.E., Wall, R. and French, N.P., 2000. *Vet. Parasitol.,* **92**: 97–105. https://doi.org/10.1016/S0304-4017(00)00277-6

Quinelato, S., Golo, S.P., Perinotto, M.S.W., Sá, A.F., Camargo, G.M., Angelo, C.I., Moraes, M.L.A. and Bittencourt, R.E.P.V., 2012. *Vet. Parasitol.,* **190**: 556–565. https://doi.org/10.1016/j.vetpar.2012.06.028

SAS, 1997. *SAS/STAT software: Change and enhancements through release 6.12.* SAS Institute, Cary, NC.