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Susceptibility of *Sphenarium purpurascens purpurascens* (Orthoptera: Pyrgomorphidae) instars to a commercial strain of *Metarhizium acridum* (Hypocreales: Clavicipitaceae) in Michoacan, Mexico

Susceptibilidad de estadios de *Sphenarium purpurascens purpurascens* (Orthoptera: Pyrgomorphidae) a una cepa comercial de *Metarhizium acridum* (Hypocreales: Clavicipitaceae) en Michoacán, México

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Abstract: The objective of this study was to evaluate the performance of a commercial strain of the entomopathogenic fungi *Metarhizium acridum*, on the different instars of the grasshopper pest *Sphenarium purpurascens purpurascens,* as well as its sporulation via bioassays under laboratory conditions, in Michoacan, Mexico. The most susceptible instars were nymphs one, two and four, with an average survival time of less than 6.91 days. The least susceptible was the adult, with a survival time of 8.36 days. The instars with the highest *post mortem* sporulation were nymphs one, two and three. A moderate negative association was registered between *S. p. purpurascens* and the sporulation of *M. acridum,* in advanced instars (nymph 4 and adult), in which sporulation was lower compared to early instars. All instars showed survival rates below 5 %, so *M. acridum* can be considered to be a viable option for controlling this pest in the field.

Keywords: Grasshopper pest, entomopathogenic fungi, sporulation, Pyrgomorphidae, *Metarhizium acridum*, Mexico.

Resumen: El objetivo de este estudio fue evaluar el desempeño de una cepa comercial del hongo entomopatógeno *Metarhizium acridum* en diferentes estadios del saltamonte plaga *Sphenarium purpurascens purpurascens,* así como su esporulación a través de bioensayos bajo condiciones de laboratorio, en Michoacán, México. Los estadios más susceptibles fueron las ninfas uno, dos y cuatro, su tiempo de supervivencia promedio fue menor de 6,91 días. El menos susceptible fue el adulto, con un tiempo de supervivencia de 8,36 días. Los estadios con mayor esporulación *post mortem* fueron ninfas uno, dos y tres. Se registró una asociación negativa moderada entre los estadios de *S. p. purpurascens* y la esporulación de *M. acridum*, en estadios avanzados (ninfa 4 y adulto), en los cuales la esporulación fue menor en comparación con los estadios inmaduros. Todos los estadios mostraron tasas de supervivencia por debajo del 5 %, por lo tanto *M. acridum* se considera una opción viable para controlar esta plaga en el campo.

Palabras clave: Saltamontes plaga, hongos entomopatógenos, esporulación, Pyrgomorphidae, *Metarhizium acridum*, México.

Introduction

The grasshopper *Sphenarium purpurascens* Charpentier, 1842 (Orthoptera: Pyrgomorphidae) is one of the most common and abundant Orthoptera in Mexico. It is widely distributed across much of the center and south of the country (Castellanos-Vargas and Cano-Santana 2009). This species feeds on and harms a great variety of crops like maize (*Zea mays* L.) (Tamayo-Mejía 2009), bean (*Phaseolus vulgaris* L.), squash (*Cucurbita* sp.), oats (*Avena sativa* L.), barley (*Hordeum vulgare* L.), wheat (*Triticum* sp.), alfalfa (*Medicago sativa* L.), clover (*Trifolium repens* L.), peas (*Cicer arietinum* L.), and various grasses (SENASICA 2012), hence being recognized as an economically important species (Anaya-Rosales *et al.* 2000).

To control this and other grasshoppers, insecticides like cypermethrin, malathion, methyl parathion, chlorpyrifos, diazinon, dimethylphosphate, deltamethrin, lambda cyhalothrin and fipronil are commonly used (Forestales and Ebano 2005), having high economic and environmental costs (Neme *et al.* 2010). The sale and distribution of many of these products are forbidden in other countries because of their potential harm to the environment (Bejarano 2001).

There are alternative methods for grasshopper control, such as biocontrol through entomopathogenic fungi (EPF), which are microorganisms infecting a great variety of arthropod groups, found in many ecosystems (Dent 1999). The use of EPF represents minimal risk to humans, domestic animals and the environment (Goettel and Johnson 1991), which is why their management as bioinsecticides to control grasshoppers and locusts is sanitary and environmentally viable (Lomer *et al.* 2001). Most commonly used EPF in a commercial scale include *Beauveria bassiana* (Bals.-Criv.) Vuill, 1912 (Hypocreales: Clavicipitaceae); *Metarhizium anisopliae* (Metchnikoff) Sorokin, 1883 (Hypocreales: Clavicipitaceae); and *Metarhizium acridum* (Driver and Milner, Hypocreales: Clavicipitaceae) (Rodríguez and Lecuona 2002; Pariona *et al.* 2007; Bischoff *et al.* 2009).

A relevant example of the use of EPF in Mexico is the CHE-CNRB 213 strain of *M. acridum*, isolated by the National Reference Center for Biological Control and used in the biocontrol program of the locust *Schistocerca piceifrons piceifrons* (Walker, 1870) (Orthoptera: Acrididae) in Socorro Island, Revillagigedo (Hernández-Velázquez *et al.* 1997). Commercial formulations based on this strain are currently available for the biocontrol of grasshoppers and locusts. Nevertheless, its pathogenicity and virulence for the control of species of Orthoptera other than the one from which it was isolated must be evaluated. For example, most strains of *B. bassiana* tend to show high virulence over its original host, or on closely related species, but there are exceptions (Feng *et al.* 1994).

In order for EPF to perform efficiently as bioinsecticides, it is recommended to evaluate the susceptibility of local insect populations at different development stages, so they can be used efficiently at the most critical stages on the field (Espinel and Torres 2009). In this context, there are few studies evaluating the susceptibility of development stages of Orthoptera towards pathogen agents and the subsequent sporulation in dead individuals, as well as taking into account the biology, ecology and population dynamics of the pest insect (Barrientos-Lozano and Almeguer-Sierra 2009).

Sphenarium purpurascens has an average life cycle of 252.4 days (Serrano-Limón and Ramos-Elorduy 1989). It presents four stages of postembryonic development before reaching adulthood (Cheaírez-Hernández and Gurrola-Reyes 2009). Adults appear gradually from August and they all die between December and January, leaving the ootheques buried in the ground for the next season (Márquez-Mayaudón 1968; Cano-Santana and Oyama 1992).

In Michoacan, Mexico, agricultural authorities recorded an average of 65 individuals per m^2 during 2014 (CESAVEMICH, personal communication, 11 May 2015), leaving important economic damage to many farmers in the region, particularly in maize crops. The economic threshold of *S. purpurascens* in maize is estimated at five nymphs/m^2 within the crop and 15 in borders and grasslands (CESAVEG 2007).

As part of a larger project aimed at generating strategies and recommendations for farmers dealing with grasshopper pests in maize crops, the objective of this study was to evaluate the performance of a commercial formulation of *M. acridum* on different stages of *S. p. purpurascens* through laboratory bioessays.

Materials and methods

Viability of the commercial product. The commercial strain was formulated on diatomaceous earth. The viability of the product was determined by the Vélez *et al.* (1997) method, which considers conidia density, their viability at 16 hours after water suspension and purity.

Collection and maintenance of biological material. The grasshoppers used were collected with an entomological sweep net in Erongarícuaro, Michoacán (19º31'23" - 19º31'13"N and 101º42'32" - 101º42'19"W). They were confined in one-liter plastic containers with modified lids that had a circular cutout. A one mm diameter mesh was placed in the cutout to allow for gaseous exchange, in order to transport them alive to the laboratory.

The grasshoppers were kept in quarantine in the containers for eight days at a density of ten individuals. They were fed with lettuce disinfected with sodium hypochlorite at 5 % for 20 minutes and rinsed with distilled water, as well as oat flakes. Excretes were removed every other day. All nymphal stages of *S. p. purpurascens* were taken into account: nymph one (N1), two (N2), three (N3), four (N4) and adult. These were identified through measurements of the length of the individuals; N1 with a length of 5 - 6.9 mm, N2 of 7 - 8.9 mm, N3 of 9 - 11 mm, N4 of 14 - 18 mm, and the adult with a lenght of 18.8 - 21.2 mm (Serrano-Limón and Ramos-Elorduy 1989; CESAVEG 2003). All individuals with lenghts outside these ranges were discarded.

Bioassays. The design of bioassays was carried out following the present protocol: each instar of *S. p. purpurascens* was exposed through a ten-second-long immersion (Cañedo and Ames 2004) in a solution with 2.5×10^6 conidia/mL of the commercial strain (dosage recommended by the manufacturer) in distilled water with 0.01 % of Tween 80® dispersant. The density was adjusted according to the previously obtained percentage of viability. A control for each of the stages was carried out, which consisted of sterile distilled water with 0.01 % Tween 80®. Each treatment was repeated five times and each replicate included ten grasshoppers, totaling 500 experimental units. Subsequently the insects were placed in individual plastic containers, with a modified lid in order to allow airflow. The grasshoppers were fed on the aforementioned diet, every other day post-application. Survival was registered daily until day 14.

Each dead grasshopper was placed in an individual humid chamber, which consisted of a 3.5 x 4.5 cm plastic container with a lid and filter paper in the bottom, dampened every other day with 200 µl of distilled water. The humid chambers were incubated at 27 °C in order to induce the sporulation of EPF on the insect. The chambers were checked daily for ten days to record sporulation.

Statistical analyses. To evaluate the effect of the commercial strain of *M. acridum* over the instars of *S. p. purpurascens*, a comparison was made between the total accumulated survival rates of the instars exposed to *M. acridum* vs. the control subjects. To complement this, the survival rates of each instar exposed to *M. acridum* were compared with the control subjects. To determine the most and least susceptible stages to the commercial product, a comparison between survival rates between the different treated instars, and the survival rates of the different control subjects were performed separately. The comparison of survival rates was done using the Kaplan-Meier log-rank method (Kaplan and Meier 1958; Tolley *et al*. 1996). The averages of grasshoppers with or without sporulation between the different instars were compared by a Bonferroni test (Westfall and Young 1989). To find out the association between instars and sporulation, a Spearman correlation coefficient was used (Martínez-Ortega *et al.* 2009). All statistical procedures were carried out using the SPSS® software version 22 (SPSS Inc. 2013).

Results

The commercial strain of *M. acridum* presented a density of 2 x 10^7 conidia/mL, similar to that reported by the manufacturer, a viability of 56.34 %, and a purity of 96.68 %.

Regarding the susceptibility of the stages of *S. p. purpurascens,* the rates of survival time between the commercial strain and the control subject presented significant differences globally (χ^2 = 398.49, d.f. = 1, P < 0.001), as well as when comparing the effect of the strain on each stage with its respective control subject (χ^2 = 395.52, d.f. = 1, P < 0.001).

N1, N2 and N4 were the most susceptible stages, showing significant differences with N3 and the adult (Fig. 1, Table 1). The survival time of N3 individuals was statistically lower in comparison with the adult (Fig. 1, Table 1). The differences in survival time of all nymphal stages in relation to the adults were significant. It was observed that survival of grasshoppers in nymphal instars decreased more than 50 % between days 6 and 8 post-treatment. The maximum survival time in adults was 14 days, with a survival rate of 5 %, in comparison with the survival time of nymphs, which was less than 11 days and with a survival rate ≤ 2.32 %. The survival time of grasshoppers in the control treatments of the different instars did not differ statistically between each other (Fig. 1, Table 1).

Table 1. Pairwise comparison (Kaplan-Meier) of survival curves with log-rank test of different stages of *Sphenarium purpurascens purpurascens* individuals inoculated with a commercial strain of *Metarhizium acridum*. N1- nymph one; N2 – nymph two; N3 - nymph three; N4 – nymph four.

Treatment	Instar	Survival time (days)	Standard error	Pairwise comparison
	N1	6.42	0.18	A
M. acridum	N2	6.91	0.25	A
	N3	7.46	0.25	B
	N4	6.00	0.32	A
	Adult	8.36	0.49	C
	N1	10.40	0.30	A
Control	N2	10.70	0.17	A
	N3	11.68	0.42	A
	N4	9.86	0.15	A
	Adult	12.78	0.45	A

Different letters show statistical differences (P < 0.05).

The highest sporulation rate was obtained in N2 and N3, both with 80.43 %, and N1 with 75.5 % of individuals, being statistically different from N4 and adults. These two stages were the ones with lowest sporulation rates in comparison with N1, N2 and N3 (Fig. 2, Table 2). The association level between instars and sporulation rates was moderate (r = -0.47, P = 0.018). It was observed that in N4 and the adult sporulation of *M. acridum* was low (50 %) (Fig. 2). The grasshoppers from control groups did not sporulate.

Discussion

The most susceptible stages of *S. p. purpurascens* to *M. acridum* were N1, N2 and N4, as they presented the lowest survival times. The adults were the least susceptible stage. The differences in survival times between stages can be attributed to chemical differences such as changes in the constitution of the proteins forming the cuticle, which are essential for the germination of conidia (Hegedus and Khachatourians 1995), as well as the different enzymes secreted by the immune system of the insect (Khachatourians 1996). N1, N2, and N3 were the stages with the highest accumulated sporulation percentage. All stages treated with *M. acridum* presented a very low survival rate, less than 4 %. Nevertheless, it was observed that sporulation was lower in more advanced instars of *S. p. purpurascens.*

There is little information available on the susceptibility of the different instars of *S. purpurascens* to EPF. Nonetheless, there are studies about the susceptibility of some stages. Vázquez *et al.* (2016) evaluated the susceptibility of N2 and N4 of *S. purpurascens* under laboratory conditions to four densities of *B. bassiana.* It was determined that N4 was the most susceptible instar, presenting a survival rate of 0 % at the sixth day post-treatment, in comparison to N2, which reached this rate on the seventh day. These results coincide with the findings of this study, although the differences were not statistically significant.

A field study reported that adults of *S. purpurascens* were less susceptible to *Metarhizium anisopliae* (Metchnikoff) Sorokin, 1883 in comparison to N2 and N3. Nymphs had a survival rate of 15 days post-application, compared to the adults with 35 days (Tamayo-Mejía 2009).

As well as knowing the most susceptible stage of the pest insect, it is necessary to understand its population dynamics

Table 2. Pairwise comparison (Bonferroni test) of *Sphenarium purpurascens purpurascens* specimens sporulated by *Metarhizium acridum*. N1- nymph one; N2 – nymph two; N3 - nymph three; N4 – nymph four.

Instar	Sporulated individuals	Standard error	Pairwise comparison
N1	0.76	0.06	A
N2	0.79	0.06	A
N3	0.86	0.05	A
N4	0.49	0.07	B
Adult	0.49	0.06	B

Different letters show statistical differences (P < 0.05).

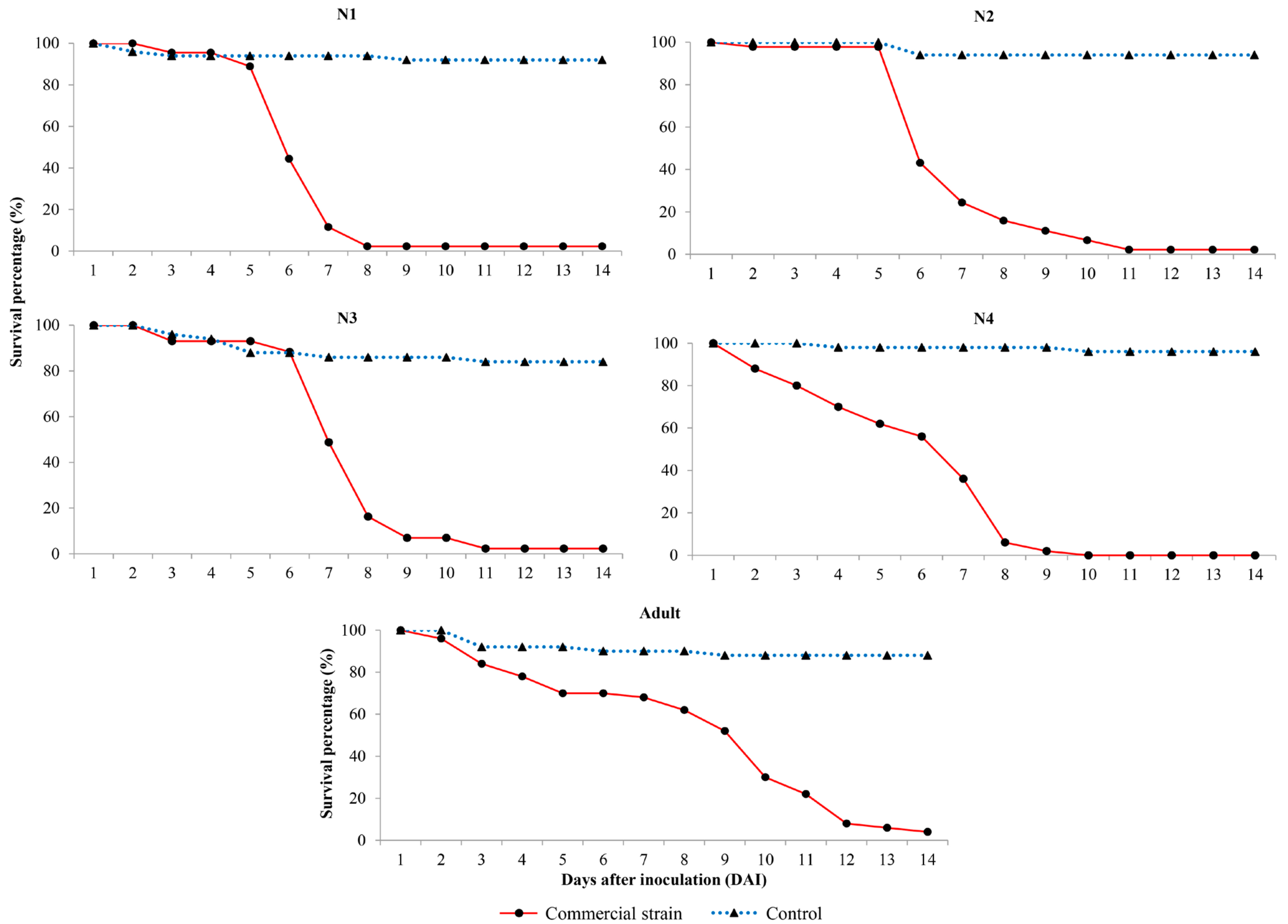

Figure 1. Accumulated survival percentage of *Sphenarium purpurascens purpurascens* instars inoculated with a comercial strain of *Metarhizium acridum* compared with control. N1, nymph one; N2, nymph two; N3, nymph three; and N4, nymph four.

and its ecological interactions with biotic and abiotic factors, as they regulate population growth (Barrientos-Lozano and Almeguer-Sierra 2009). In a field study, Tamayo-Mejia (2009) observed a 50 % death rate of *S. pupurascens* in N1 and N2 stages due to biotic and abiotic factors. From N3 to adults, population densities remained constant. Even though these results are not comparable, it is possible that field application of *M. acridum* in early stages, in combination with those factors, could eliminate most individuals.

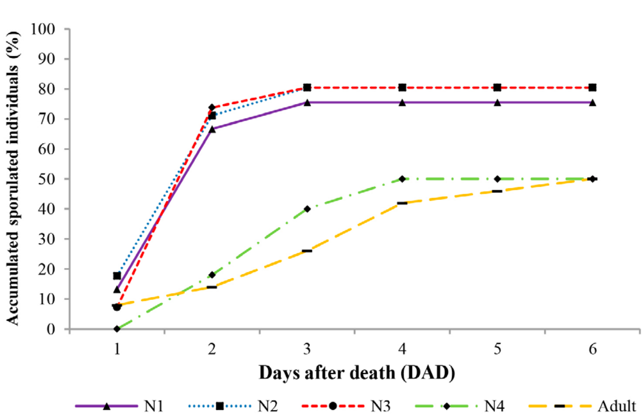

Figure 2. Accumulated post-mortem sporulation percentage of *Metarhizium acridum* on *Sphenarium purpurascens purpurascens*. N1, nymph one; N2, nymph two; N3, nymph three; N4, nymph four.

Regarding sporulation of *M. acridum* over *S. p. purpurascens*, N1, N2 and N3 were the stages with the highest rates. Upon performing a correlation between the different stages and the sporulation of *M. acridum*, a negative association was found. It was observed that in the most advanced instars of *S. p. purpurascens*, N4 and adult, sporulation was low. One of the possible explanations is the reduction of essential nutrients for conidia growth (Gottwald and Tedders 1984).

Field experiments are required to evaluate the results of the present study. It is recommended to start the application of *M. acridum* on the field when *S. p. purpurascens* is at N1 and N2, which allows a greater probability of sporulation in dead individuals. In addition, at these stages of development, grasshoppers are found at the borders of maize crops (Quesada-Béjar *et al.* 2017). It is equally recommended to perform a second application on N4, as it is one of the most susceptible stages.

Conclusions

This study determined that the inoculation of the commercial strain CHE-CNRCB 213 of *M. acridum* resulted in very low survival rates of *S. p. purpurascens* under controlled conditions, therefore being a viable option to regulate its populations. The most convenient instars for application of *M. acridum* on *S. p. purpurascens* are N1, N2 and N4.

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Author contribution

Venecia Quesada-Béjar: carried out insect collections, lab experiments, statistical analyses and wrote the manuscript.

Miguel Nájera-Rincón: carried out experimental design, trained Venecia Quesada-Béjar on field collections and lab techniques and reviewed and corrected the manuscript.

Enrique Reyes-Novelo: advised on the experimental design, reviewed the results and corrected the manuscript.

Carlos E. González-Esquivel: designed and supervised the study, reviewed the results, reviewed, corrected and translated the manuscript.