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Differential Pathogenicity of *Metarhizium anisopliae* and the Control of the Sugarcane Root Spittlebug *Mahanarva fimbriolata*

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ABSTRACT

In order to assess the effectiveness of Metarhizium anisopliae *var.* anisopliae *(Metschnikoff) Sorokin isolates in controlling the sugarcane root spittlebug* Mahanarva fimbriolata *(Stal) (Hemiptera: Cercopidae), nine isolates obtained from a single geographical region were studied. 'Confirmed cumulative' and 'corrected cumulative' spittlebug mortality rates were measured for each of the isolates. Based on the confirmed mortality curve, the isolates URM5946, URM5951 and URM6033 were considered to be potentially the most effective in a biological control program for* M. fimbriolata.

Key words: biological control, entomopathogenic fungus, *Metarhizium anisopliae*, *Mahanarva fimbriolata*.

INTRODUCTION

In Brazil, sugarcane monoculture forms the basis of the sugar export and biofuel industries (Alves et al., 2008). The root spittlebug *Mahanarva fimbriolata* (Stal) (Hemiptera: Cercopidae) causes serious economic damage to this crop in regions where harvesting is mechanized, especially in the Southeast and in some states of the Midwest and Northeast (Dinardo-Miranda et al., 2006). This necessitates to develop the efficient ways to control this pest.

Biological control of the spittlebug using the fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin may provide an alternative to the use of chemical insecticides. Additional benefits of using this particular pathogen are that it is also active against the borer *Diatraea saccharalis* (Fabricius) but does not affect some of the natural enemies of the spittlebug such as the wasp *Salpingogaster nigra* (Schiner) and predator ant *Pheidole genalis* (Borgmeier) (Mendonça and Mendonça, 2005).

Several studies have been conducted that focus on the isolation and screening of entomopathogenic fungi which may be effective in controlling the pest insects (Entz et al., 2008; Anand et al., 2009). For example, the pathogenicity of isolates of *M. anisopliae* from different hosts and regions of Brazil have been tested against the spittlebug *M. fimbriolata* (Loureiro et al., 2005; Macedo et al., 2006). Because of the current importance of this pest in sugarcane cultivation, the use of *Metarhizium* is likely to increase for controlling

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the pest. Thus, this study focused on the selection of isolates of *M. anisopliae* var. *anisopliae* with high pathogenicity for *M. fimbriolata* nymphs.

MATERIAL AND METHODS

Source of *Metarhizium anisopliae* var. *anisopliae* **isolates**

The isolates of *M. anisopliae* var. *anisopliae* (URM5946, URM5947, URM5948, URM5949, URM5950, URM5951, URM5952, URM6033 and URM6034) were obtained from the nymphs and adults of *M. fimbriolata* in Tangará da Serra city, Mato Grosso, Brazil, 14º47'47"S - 57º49'07"W. These isolates were deposited in the URM Culture Collection, Department of Mycology, Federal University of Pernambuco, Recife, PE, Brazil.

Collection of *Mahanarva fimbriolata* **nymphs**

The nymphs were collected from the sugarcane crops without pesticides and mycoinsecticide in the city of Tangará da Serra, Mato Grosso, Brazil. They were captured with the help of entomological forceps and placed in glass containers containing a few pieces of cane leaf and transported to the laboratory in a polystyrene box.

Bioassays to evaluate the pathogenicity of *Metarhizium anisopliae* var. *anisopliae*

Three separate tests were carried out, each involving a different sub-set of isolates. A randomized design was used in a split plot, subdivided in time (1, 2, 3, 4, 5, 6 days survival), with nine isolates plus one control prepared in the plots and the evaluation days in the subplots, replicated 5 times.

Bioassay 1 involved the isolates URM5946, URM5952, URM6034; bioassay 2 involved the isolates URM5947, URM5948, URM5949, URM5950 and bioassay 3 involved the isolates URM5951, URM6033.

In each case the confirmed cumulative and corrected cumulative mortality occurring after inoculation was measured. The confirmed mortality corresponded to the percentage of insects on which fungal sporulation could be confirmed and the corrected mortality was calculated by the formula of Abbott (1925), removing the individuals that died due to natural causes (control) and calculating the death proportions of those which were subjected to specific fungus concentrations (total mortality).

The isolates were inoculated into potato dextrose agar (PDA) contained in Petri dishes and incubated in BOD chamber at 26 ±. 1ºC for ten days. Subsequently, the suspensions of conidia were prepared in sterile distilled water + Tween 80 (0.03% v/v) at concentration of $1x10^8$ conidia/mL and a control treatment with sterile distilled water + Tween 80 (0.03% v/v). Spittlebug nymphs were immersed in conidial suspensions for ten seconds and then transferred to transparent plastic boxes (34 cm x 22 cm x 12 cm) containing two leaves of sugarcane (8 cm in length) washed with sterile distilled water and kept at 26 ± 1ºC, 12h photoperiod and 70 ± 10% Relative Humidity (RH). For each treatment, five plastic boxes, each containing ten nymphs and totaling fifty nymphs, were used. Mortality was monitored daily until the sixth day after immersion and dead nymphs were placed individually in Petri dishes with a piece of cotton cloth, setting a moist chamber, and taken again to the BOD chamber to verify the mycelial growth and conidiagenesis on the corpses.

Statistical analysis

Analysis of covariance (ANCOVA) was used to assess the differences in the cumulative mortality data (confirmed and corrected) following an ARCSINE $\sqrt{(x/100)}$ transformation. The ANCOVA technique allowed to determine the differences in slope (parallelism) and level (coincidence) between the regression curves obtained in each test. In the event that no parallelism ($p < 0.05$) occurred, the isolates were 2 x 2 tested to find the differences in slope. For the pairs which had parallel curves ($p > 0.05$), the difference between the intersections on the Y axis were tested. In the cases where there was parallelism ($p > 0.05$), the difference in Y intersection was tested and when differences were significant, the Tukey test was used to identify which of these differences in intersection were significant (Zar, 2009).

RESULTS

There was no parallelism in regression lines obtained for the isolates studied in the bioassay 1 ($F_{(2, 84)} = 13.239$, $p = 0.000010$) (Figure 1a) and bioassay 2 ($F_{(3, 112)} = 2.707$, $p = 0.0487$) (Figure b), indicating the existence of differences in the confirmed cumulative mortality between the treatments. In the bioassay 1, the isolates

URM5946 and URM6034 caused the maximum confirmed mortality rate and did not differ from each other, having regression lines with similar slope ($F_{(1, 56)} = 1.194$, $p = 0.279$, $b_{URM5946} = 0.1688$, $b_{URM6034} = 0.1359$), but showing differences of intersection ($F_{(1, 57)} = 11.300$, $p = 0.0014$). Differences in the slope occurred between the isolates URM5946 and URM5952 ($F_{(1, 56)} = 31.022$, $p = 0.000001$) and the isolates URM6034 and URM5952 ($F_{(1, 56)} = 15.013$, $p = 0.0003$), whereas the isolated URM5952 presented the lowest regression coefficient ($b_{URM5952} = 0.0381$) (Figure 1a).

In bioassay 2, the isolate URM5948 caused the maximum confirmed mortality compared to the isolate URM5947, presenting significant differences between the regression coefficients ($F_{(1, 56)} = 7.009$, $p = 0.010$, $b_{URM5948} = 0.1524$, $b_{URM5947} = 0.0781$). The mortality recorded was similar for the isolates URM5948, URM5949 and URM5950, showing regression lines with similar slope ($F_{(2, 84)} = 0.90$, $p = 0.410$, $b_{URM5949} = 0.1264$, $b_{URM5950} = 0.1157$), though there were no observed differences of intersection ($F_{(2, 86)} = 1.1856$, $p = 0.310$) (Figure 1b).

There was parallelism of regression lines obtained for the isolates studied in the bioassay 3 ($F_{(1, 56)} = 1.755$, $p = 0.190$, $b_{URM5951} = 0.1829$, $b_{URM6033} = 0.1368$), indicating the existence of a pattern of confirmed cumulative mortality for the isolates URM5951 and URM6033 (Figure 1c). These isolates reached 50% of confirmed cumulative mortality on the sixth day, and as there were no differences between the regression coefficients, there were also no differences between the curves the intersections ($F_{(1, 57)} = 0.516$, $p = 0.475$).

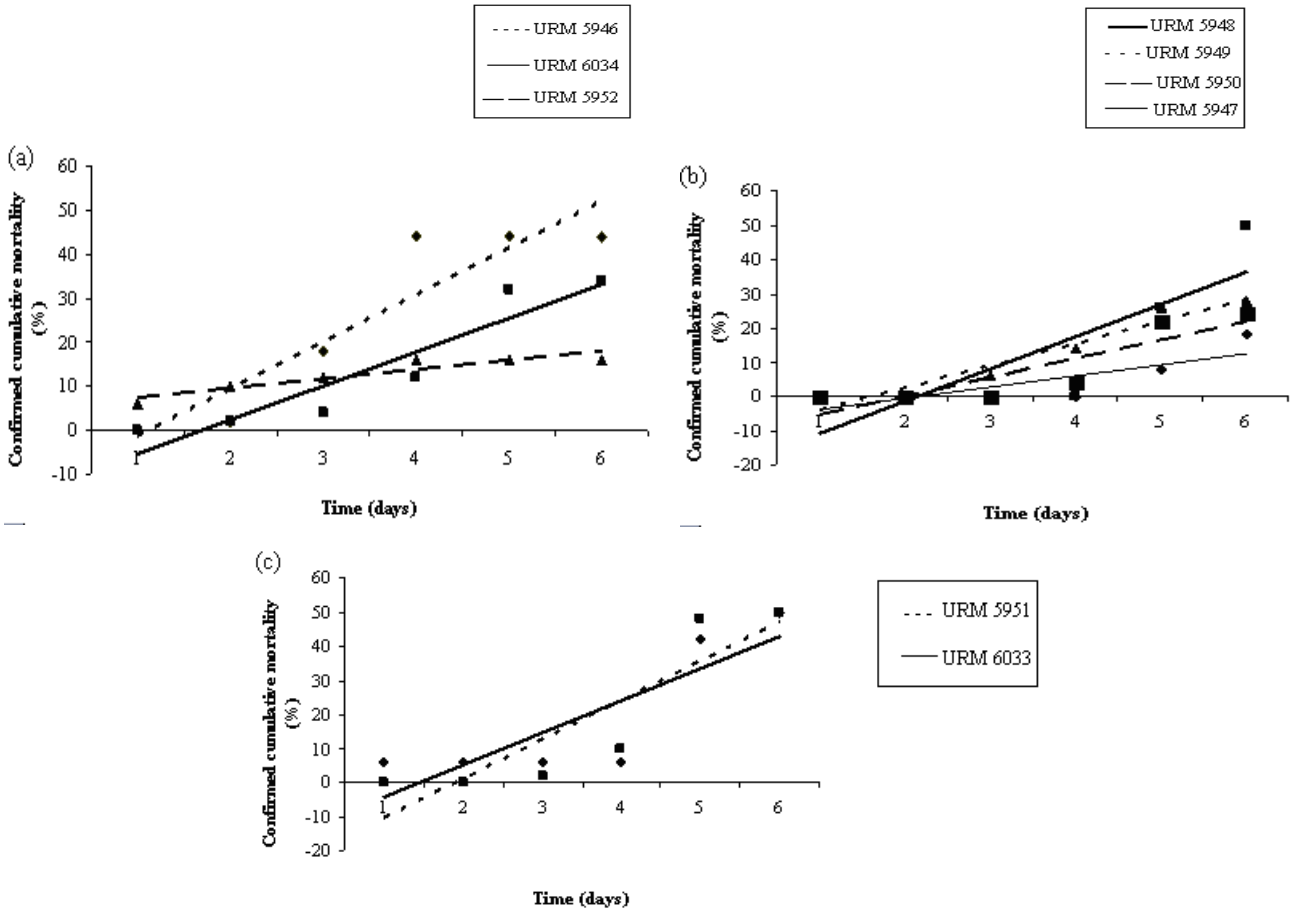
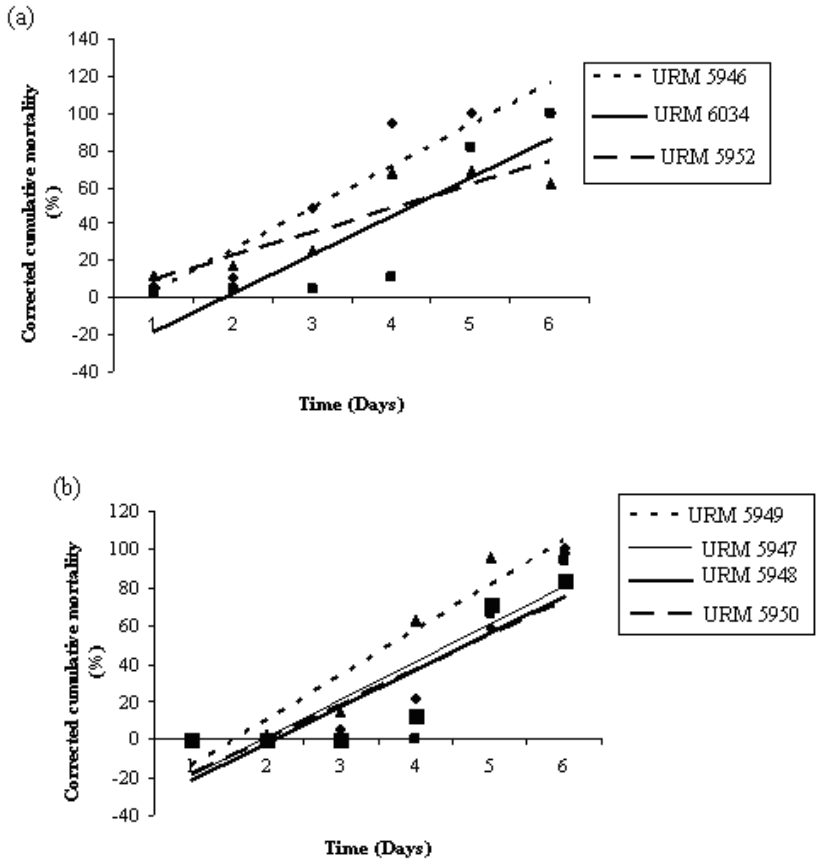


Figure 1 - Confirmed cumulative mortality (%) of *Mahanarva fimbriolata* nymphs over six days after application of different isolates of *Metarhizium anisopliae* var. *anisopliae*. a) Bioassay 1: ---♦--- URM5946; ▬▬■▬▬ URM6034; ─ ─▲─ ─ URM5952. b) Bioassay 2: ──■── URM5948; ---▲--- URM5949; ─ ─■─ ─ URM5950; −♦− URM5947. c) Bioassay 3: ---■--- URM5951; ──♦── URM6033. Original data, for statistical analysis were transformed into ARCSINE √ (x/100).

The insect resistance to the isolate URM5952 was lower initially, followed by the isolates URM5946 and URM6034, but only the latter two showed a good efficiency in controlling the nymphs (Figure 1a). In the bioassay 2, the nymphs initially showed higher susceptibility to the isolate URM5949 and lower to the isolate URM5948, but both reached similar final efficiency (Figure 1b). In the bioassay 3, despite the isolates URM5951 and URM6033 having similar effects, the nymphs showed increased susceptibility to the isolate URM6033 initially (Figure 1c). In the control treatment, the nymphs, which died during the days of evaluation, did not present fungus sporulation, hence, the confirmed cumulative mortality was zero in the three bioassays.

The efficiency of the isolates studied in the three bioassays was also presented in corrected form. There was no parallelism of regression lines ($F_{(2, 84)} = 5.163$, $p = 0.0077$) in the bioassay 1 (Figure 2a). The isolates efficiency was higher in the corrected form than in the confirmed one, but in both the cases, the isolates URM5946 and URM6034 stood out (Figure 1a and Figure 2a).

There was parallelism of regression lines obtained for the isolates studied in the bioassay 2 ($F_{(3, 112)} = 1.497$, $p = 0.210$, $b_{URM5947} = 0.2025$, $b_{URM5948} = 0.2141$, $b_{URM5949} = 0.2354$, $b_{URM5950} = 0.1630$) (Figure 2b) and in the bioassay 3 ($F_{(1, 56)} = 0.142$, $p = 0.708$, $b_{URM5951} = 0.2613$, $b_{URM6033} = 0.2321$) (Figure 2c), indicating the existence of a pattern of corrected cumulative mortality. In the bioassay 2, the isolates showed regression lines with similar slope, but differences in the intersection were observed ($F_{(3, 115)} = 7.775$, $p = 0.00009$), and the isolated URM5949 differed from the others (Figure 2b). The isolates did not differ in terms of corrected cumulative mortality in this bioassay. In the bioassay 3, the efficiency of these isolates was higher in the corrected form when compared to the confirmed one, but in both the cases, there was no significant difference between the isolates (Figure 1c and Figure 2c).



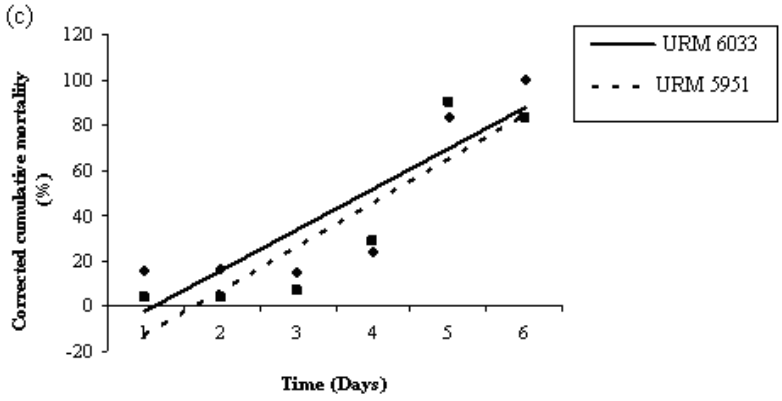


Figure 2 - Corrected cumulative mortality (%) *Mahanarva fimbriolata* nymphs over six days after application of *Metarhizium anisopliae* var. *anisopliae*. a) Bioassay 1: ---♦--- URM5946; ▬▬■▬▬ URM6034; ─ ─▲─ ─ URM5952. b) Bioassay 2: ---▲--- URM5949; ──♦── URM5947; ▬■▬ URM5948; ─ ─■─ ─ URM5950. c) Bioassay 3: ---■--- URM5951; ▬♦▬ URM6033. Original data, for statistical analysis were transformed into ARCSINE √ (x/100).

Confirmed mortality curves were used as criteria for the selection of the isolates. Thus, the isolates URM5946 (bioassay 01), URM5951 and URM6033 (bioassay 03) showed promising features to be used in a biological control program of *M. fimbriolata*. The fungi that caused low confirmed mortality and high corrected mortality are not recommended for control of *M. fimbriolata* nymphs.

DISCUSSION

The nine isolates of *M. anisopliae* var. *anisopliae* studied caused low to medium mortality values after six days (16-50%). The selection of entomopathogenic fungi often involves the isolates from different hosts and geographical regions (Ihara et al., 2001; Samuels et al., 2002; Loureiro et al., 2005) because it is believed that exotic variants might be more virulent. However, Loureiro et al. (2005) found low confirmed mortality when compared the 27 isolates obtained from the spittlebugs across different regions of Brazil and only two isolates showed mortality between 80 - 88% on the sixth day.

Other studies involving the pathogenicity of *M. anisopliae* tested against various insects of the order Hemiptera, have presented very heterogeneous results. *M. anisopliae* caused 87.1% confirmed mortality against *Oncometopia facialis* (Signoret) (Hemiptera: Cicadellidae), vector of citrus variegated chlorosis (Pria Júnior et al., 2008). With the bug *Cyrtomenus bergi* (Froeschner) (Hemiptera: Cydnidae), the confirmed mortality did not exceed 55% (Jaramillo and Borgemeister, 2006). For the scale-borer *Dysmicoccus texensis* (Tinsley) (Hemiptera: Pseudococcidae) most isolates of *M. anisopliae* caused confirmed mortality between 30 and 50% (Andaló et al., 2004). These studies used the analysis of variance (ANOVA) and the means test of mortality data obtained on the last day of evaluation to determine the most efficient isolates. In the present study, the analysis of covariance enabled to identify the most virulent *M. anisopliae* var. *anisopliae* isolates by testing the parallelism between the curves as a function of time. Thus, the most virulent isolates URM5946, URM5951 and URM6033 were those that caused mortality in less time and with good final efficiency.

The confirmed mortality data analyzed over time showed differences in insect susceptibility for each isolate. Except for the isolates URM5952 (bioassay 1) and URM5947 (bioassay 2), nymph susceptibility was directly related to the isolates that caused highest rate of final confirmed mortality. This fact usually is associated with virulence reflecting pathogen's ability to block the natural resistance of the insect, invade and multiply in the host tissues (Thomas and Elkinton, 2004).

Confirmed mortality differentiated between the isolates of *M. anisopliae* var. *anisopliae* concerning their efficiency in controlling *M. fimbriolata* nymphs in the bioassays 1 and 2.

Variations in confirmed mortality possibly occurred due to pathogen's genetic variability, the use of a heterogeneous insect population or the stress suffered by these insects when removed from the field. It is possible that some insects did not show sporulation of the entomopathogen due to not having been killed by the action of the fungus and others died before starting the fungus infection. According to Loureiro et al. (2005), in some cases a generalized sepsis caused by bacteria might have occurred which interfered with the fungus' vegetative growth inside the insect.

The isolates that caused higher confirmed cumulative mortality rate had moderate virulence, which is an important feature when considering the use of pathogens, using the strategies of inoculative or incrementing introduction.

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