

## Effect of two dosages of *Metarhizium anisopliae* var. *acidum* against *Rhammatocerus schistocercoides* Rehn<sup>(1)</sup>

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**Abstract** – The fungus *Metarhizium anisopliae* var. *acidum*, strain CG 423, was tested under field conditions against the gregarious grasshopper *Rhammatocerus schistocercoides* (Rehn) (Orthoptera: Acrididae). Conidia formulated in a racemic mixture of soybean oil and kerosene were sprayed under field conditions using an ultralow-volume hand-held atomizer Ulva Plus adjusted to deliver 2.9 L/ha. Bands composed of 2<sup>nd</sup> instar nymphs were treated with either  $5.0 \times 10^{12}$  or  $1.0 \times 10^{13}$  viable conidia/ha. The number of insects in each band was estimated at day one following spraying and by the end of the field trial (15 to 16 days post-treatment). Reductions in population size reached, in average, 65.8% and 80.4% for bands treated with the higher and lower dosage, respectively. For both dosages, total mortality rates of insects collected at two days post-application, and kept in cages for 14 days under lab conditions, showed no significant differences as compared to that obtained with insects collected immediately after spraying. Healthy insects were fed to native grasses sprayed on the field with  $1.0 \times 10^{13}$  viable conidia/ha. Mortality levels of the nymphs fed on grasses collected two and four days post-application were not affected when compared to nymphs fed on grasses collected immediately following application.

**Index terms:** biological control, microbial pesticide, entomogenous fungus, grasshopper, oil formulation.

### Efeito de duas dosagens de *Metarhizium anisopliae* var. *acidum* contra *Rhammatocerus schistocercoides* Rehn

**Resumo** – O fungo *Metarhizium anisopliae* var. *acidum*, isolado CG 423, foi avaliado em condições de campo como agente de controle biológico do gafanhoto gregário *Rhammatocerus schistocercoides* (Rehn) (Orthoptera: Acrididae). Conídios formulados em uma mistura racêmica de óleo de soja e querosene foram pulverizados no campo com a utilização de um atomizador rotativo manual Ulva Plus, calibrado para aplicação de 2,9 L/ha. Bandos com ninfas de 2<sup>o</sup> estágio foram tratados com o equivalente a  $5,0 \times 10^{12}$  ou  $1,0 \times 10^{13}$  conídios viáveis/ha. O número de insetos em cada bando foi estimado no 1<sup>o</sup> dia após a aplicação e ao final do experimento (15 a 16 dias após tratamento). Reduções populacionais médias de 65,8% e 80,4% foram observadas nos bandos tratados com a maior e menor dosagem, respectivamente. Para as dosagens avaliadas, a mortalidade total de insetos coletados aos dois dias após a aplicação e mantidos em gaiolas durante 14 dias, no laboratório, não apresentou diferença significativa daquela obtida para insetos coletados imediatamente após a pulverização. Insetos saudáveis foram alimentados com gramineas nativas pulverizadas no campo com  $1,0 \times 10^{13}$  conídios viáveis/ha. As taxas de mortalidade observadas nas ninfas alimentadas com capim coletado aos dois e quatro dias após a pulverização não foram afetadas quando comparadas com ninfas alimentadas com capim coletado imediatamente após a pulverização.

**Termos para indexação:** controle biológico, pesticida microbiano, fungo entomopatogênico, gafanhoto, formulação oleosa.

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## Introduction

Although biopesticides represent approximately 1.3% of the phytosanitary products used in the world, those directed to insect control have 4.5% of the market share for insecticides (Menn & Hall, 1999). Products based on the bacterium *Bacillus thuringiensis* account for most of the sales, but those

based on entomopathogenic fungi, specially the mitosporic group, have become very promising. In Brazil, production methods of entomopathogenic fungi were developed in the 70's (Aquino et al., 1977), and are based on rice as the substrate. The yields are relatively low and, for fungi such as *Metarhizium anisopliae* var. *acridum* and *M. anisopliae* var. *anisopliae*, vary from 2.5 to  $6.0 \times 10^9$  conidia per gram under laboratory conditions (Quintela, 1994; Magalhães & Frazão, 1996). As an additional disadvantage, conidia produced are not formulated, reducing the potential for longer shelf-lives and, therefore, turning production dependent on immediate demands.

In recent years, insect resistance to chemicals, high costs of research and development of new synthetic molecules, and technological breakthroughs on production, formulation and delivery of mycoinsecticides, have expanded the commercial success of these products (Wraight & Carruthers, 1999). For example, the private company Mycotech (Butte, Montana, USA) has developed a diphasic fermentation system that allows an average yield of  $2.6 \times 10^{10}$  conidia of *Beauveria bassiana* per gram on a proprietary substrate (Johnson et al., 1992). In addition, formulations based on oils (Thomas et al., 1996; Lomer et al., 1997; Milner et al., 1997) and adjuvant emulsifiable oils (Alves, 1999; Bateman & Alves, 2000) have proved suitable in many circumstances. Despite the mentioned advances, the balance between economically feasible rates and satisfactory control levels is delicate and, in some situations, responsible for failure cases of biological control programs based on entomopathogenic fungi. Studies carried out at field conditions aiming the establishment of appropriate dosages are critical in biological control programs involving biopesticides.

Until recently, the gregarious grasshopper *Rhammatocerus schistocercoides* was considered a severe pest in the Chapada dos Parecis, Mato Grosso State, Brazil, attacking rice, corn and sugarcane (Lecoq & Pierozzi Junior, 1995a, 1995b; Miranda et al., 1996). This pest was chosen as a model system for a research project started in 1993 and aiming the microbial control of grasshoppers. Preliminary studies resulted in selection of the fungal pathogen *M. anisopliae* var. *acridum*, strain CG 423

(Moreira et al., 1996; Magalhães et al., 1997b) for deeper studies. A recent field trial performed at the permanent outbreak zone of *R. schistocercoides* resulted in outstanding mortality rate (88%), demonstrating the fungus to be well suited for control strategies of grasshopper pests (Magalhães et al., 2000b). However, it was sprayed at a very high dosage, equivalent to  $2.0 \times 10^{13}$  conidia/ha.

The objective of the present work, was to evaluate the effect of *M. anisopliae* var. *acridum*  $1.0 \times 10^{13}$  and  $5.0 \times 10^{12}$  conidia/hectare against the gregarious grasshopper *Rhammatocerus schistocercoides* (Rehn) (Orthoptera: Acrididae).

## Material and Methods

### Fungal production and formulation

Conidia of the strain CG 423 of *M. anisopliae* var. *acridum* were produced in parboiled rice according to the methodology described in Magalhães & Frazão (1996). Conidia were transported to Campos de Júlio county (Mato Grosso State) as a powder, and added to a racemic mixture of soybean oil plus kerosene prior to field application.

### Selection of grasshopper bands

Search for grasshopper bands was carried out for three days before first fungal application. Different sites were visited and all bands mapped. The accurate position of each band was recorded on a Global Positioning System. Nine bands, considered as having adequate sizes (between 120 and 600 m<sup>2</sup>), and located in areas far away from roads and, as much as possible, distant from other bands were selected. Information regarding the experimental area and procedures related to monitoring of grasshopper bands were previously reported (Magalhães et al., 2000b).

### Bioinsecticide application

Red nozzle output and deposition tests using a hand-held ULV spinning-disk atomizer, powered by six 1.5 V batteries, indicated the output to be 70 mL per minute, and the adequate spacing between swatches to be 4 m. Walking speed of 1 m/s and emission height of 1 m were adopted. Volume application rate was equivalent to 2.9 L/ha, and field dosages of  $5.0 \times 10^{12}$  or  $1.0 \times 10^{13}$  viable conidia/ha. For each dosage, 3 bands with most nymphs being on the 2<sup>nd</sup> instar were treated. Bands treated with the low dosage ( $5.0 \times 10^{12}$ ) were designated as L1, L2 and

L3, and those treated with the high dosage ( $1.0 \times 10^{13}$ ) were referred to as bands H1, H2 and H3. Spraying was limited to the surface area covered by each band, ranging from approximately 100 to 300 m<sup>2</sup> at the time of spraying. Untreated bands (U1, U2 and U3) were considered as controls. Table 1 indicates the prevailing conditions by the time spraying was performed. Prior to application, conidial viability was determined according to methodology described by Magalhães et al. (1997a), and re-assessed following spraying. Bands were daily followed in their natural environment, from 15 (low dosage bands) to 16 days post-spraying (high dosage bands).

### Measuring size and density of bands

Surface area and total number of nymphs per band were estimated at the beginning (day one) and by the end of the field trial. Surface area was determined by delimiting the band and dividing it in four different estimated density zones at day 1 post-spraying. The densities were 2,512, 696, 192, and 39 nymphs per m<sup>2</sup>, considered as extremely high, high, medium and low, respectively. Average numbers were obtained by throwing a metal barrel measuring 0.26 m<sup>2</sup> on specific density zones within bands and counting the number of captured insects. The total number of nymphs per band was determined by multiplying the surface area of each density zone by the correspondent number of nymphs per m<sup>2</sup>, and adding the values up. Since the average number of nymphs per density zones changed according to nymphal instar, number of individuals in a given band was overestimated by the end of the experiment, when band sizes had increased due to molting of nymphs to 3<sup>rd</sup> instar. Nymphal mortality due to natural factors in control bands was expected to be low, equivalent to a theoretical value of 0.86% a day (Miranda et al., 1996), which turns out to correspond to 13.8% after 16 days. This value was confirmed in a field trial carried out in 1998 (Magalhães et al., 2000b), when a mortality

rate of 13.1% was observed after 14 days. Numbers calculated at the end of the field trial for treated bands were adjusted considering the expected natural mortality (0.86% per day) and the increasing size constant (ISC) derived from observations of untreated bands. The constant (ISC = 2.19) was obtained by dividing the average number of insects in untreated bands estimated at the final and beginning of the trial. Distances daily traveled by bands were measured as well.

### Caged insects

For each conidial dosage, nymphs were collected at zero, two and four days post-spraying of the fungus. For untreated bands, insects were collected only at the beginning of the experiment. Groups of 30 insects were kept in cages and fed to native grass *Andropogon selloanus* Hackel, which was replaced every other day. Insects were daily observed and dead insects placed on a moist chamber in order to confirm if death was caused by *M. anisopliae* var. *acridum*. Insects showing the typical red coloration or external mycelial growth on the cadaver were considered as killed by the pathogen.

In a second experiment with caged insects, the persistence of the fungus *M. anisopliae* var. *acridum* when sprayed on vegetation at a dosage equivalent to  $1.0 \times 10^{13}$  conidia/ha was studied. Grass leaves collected at a sprayed site, immediately after, two and four days following spraying, were offered to healthy nymphs kept in cages. Total percentage of deaths and confirmed mortality rates were then determined. Each treatment comprised three replicates, and insects were kept under laboratory conditions for 14 days. In the last experiment, nymphs were collected 11 days post-spraying within spots of band H1/H2 presenting extremely high, high, medium and low nymphal densities. Insects were kept under laboratory conditions for seven days, then the cumulative total and confirmed mortality levels were recorded. Only one replicate was performed per class of nymphal density.

**Table 1.** Parameters prevailing during spraying of fungus *Metarhizium anisopliae* var. *acridum* against the hopper *Rhammathocerus schistocercoides* under field conditions.

Parameters	Hopper band <sup>(1)</sup>					
	L1	L2	L3	H1	H2	H3
Date	November, 29th	November, 29th	November, 29th	November, 28th	November, 28th	November, 28th
Time (h)	16:35 / 16:43 <sup>(2)</sup>	17:37 / 17:44	18:26 / 18:39	16:55 / 17:05	17:43 / 17:52	18:26 / 18:35
Temperature (°C)	27.4 / 24.9	23.0 / 23.4	22.4 / 22.4	25.9 / 24.1	23.3 / 22.6	22.4 / 22.0
Average wind speed (ms <sup>-1</sup> )	1.2 / 2.0	0.9 / 0.9	1.3 / 1.1	<0.5 / 1.3	2.9 / 1.2	0.7 / 1.2
Light intensity (lux)	227x10 <sup>2</sup> / 66x10 <sup>2</sup>	16.4x10 <sup>2</sup> / 10.9x10 <sup>2</sup>	30 / 0	261x10 <sup>3</sup> / 176x10 <sup>2</sup>	63x10 <sup>2</sup> / 20x10 <sup>2</sup>	2x10 <sup>2</sup> / 1.3
Other	Cloudy, light rain	Cloudy, light rain	Cloudy, light rain 2 minutes before spraying	Cloudy, it stopped raining 40 minutes before spraying	Cloudy	Cloudy

<sup>(1)</sup>L1, L2, L3 and H1, H2, H3: hopper bands treated with low dosage ( $5.0 \times 10^{12}$  conidia/ha) and high dosage ( $1.0 \times 10^{13}$  conidia/ha) of *M. anisopliae* var. *acridum*.

<sup>(2)</sup>Numbers before and after slash refer to prevailing conditions at the beginning and at the end of the spraying period, respectively.

## Analyses

Although there were three treated bands for each application rate at day one, the number of bands was reduced to two due to merging of bands along the period. Therefore, statistical analyses were not performed for the field trial. Regarding laboratory assays, percentage data were submitted to one way analysis of variance, and average comparisons were done with t test or Tukey at a 5% significance level. The software SigmaStat (Kuo et al., 1992) was employed for the statistical procedures.

## Results and Discussion

One day prior to application, viability of pure conidia of *Metarhizium anisopliae* var. *acridum* was 89%. Following spraying, viability of conidia formulated in the racemic mixture of soybean oil and kerosene was re-assessed and showed no reduction.

No significant differences in total mortality were observed immediately following application, two or four days post-spraying (t test;  $P \geq 0.145$ ) when the two dosages were compared under laboratory conditions (Table 2). A different trend was observed for confirmed mortality, since at immediately following application and two days post-application the averages for treatment with  $5.0 \times 10^{12}$  conidia/ha were significantly higher ( $P \leq 0.038$ ) than four days post-application.

For the low dosage, total mortality average of nymphs collected at day four post-spraying was significantly reduced (Tukey;  $P = 0.004$ ). For the high dosage, no differences were observed among averages ( $P = 0.330$ ). Regarding confirmed mortality, for both dosages the differences among averages were not significant ( $P \geq 0.080$ ). According to Vicentini & Magalhães (1996), events related to attachment, germination and penetration of strain CG 423 into the host take place in 18 hours. The results demonstrate that the invasive capability of conidia exposed to environmental constraints for at least two days is not being severely affected.

No significant differences in either total mortality or confirmed mortality under laboratory conditions were found when nymphs were fed on grasses collected immediately following application, two and four days post-spraying ( $P = 0.167$  and  $0.056$  for total and confirmed mortality, respectively), although there is a trend of grasses collected at day four to induce lower mortality levels (Table 3). During the trial period and, specially, until two days post-spraying, UV radiation and other detrimental factors did not play a major role in conidia inactivation. The oil formulation may have some contribution to this persistence, as previously reported in other studies (Moore et al., 1993; Alves et al., 1998). Besides, climatic condition at Campos de Júlio county during the field trial, characterized by mild temperatures, high relative humid-

**Table 2.** Percentage of mortality of the hopper *Rammatocerus schistocercoides* nymphs collected at various times following field application of fungus *Metarhizium anisopliae* var. *acridum* ( $5.0 \times 10^{12}$  and  $1.0 \times 10^{13}$  conidia/ha) and kept under laboratory conditions for 14 days.

Replicate <sup>(1)</sup>	Total mortality			Confirmed mortality		
	Day 0	Day 2	Day 4	Day 0	Day 2	Day 4
	$5.0 \times 10^{12}$					
1	80.0	85.2	70.0	66.7	70.4	60.0
2	85.7	93.1	50.0	71.4	65.5	23.3
3	85.7	85.2	53.3	52.4	55.6	20.0
Average <sup>(2)</sup>	83.8±1.90a	87.8±2.63a	57.8±6.19a	63.5±5.71a	63.8±4.35a	34.4±34.4a
	$1.0 \times 10^{13}$					
1	53.3	80.8	66.7	30.0	30.8	50.0
2	66.7	64.3	66.7	50.0	35.7	36.7
3	83.3	86.2	46.7	33.3	13.8	23.3
Average	67.8±8.68a	77.1±6.59a	60.0±6.67a	37.8±6.19b	26.8±6.64b	36.7±7.71a

<sup>(1)</sup>Insects from replicates 1, 2 and 3 of treatments  $5.0 \times 10^{12}$  and  $1.0 \times 10^{13}$  were collected from bands L1, L2, L3 and H1, H2 and H3, respectively. <sup>(2)</sup>In a given column, average numbers±standard error followed by the same letter are not significantly different according to t test ( $\alpha = 0.05$ ); average total and confirmed mortality rates for the control were  $21.1 \pm 7.76\%$  and  $0\%$ , respectively.

ity and intense cloudiness (Table 1), was an important factor that contributed to the satisfactory performance of the fungus. The persistence of *M. anisopliae* var. *acridum* on the foliage probably increases the level of fungal infection due to secondary pickup, but so far the role of this process was not addressed in our trials.

In the field, one band of the low dosage (L1) merged with an untreated band on December 2<sup>nd</sup> and it was not considered thereafter. Two treated bands of the high dosage (H1 and H2) merged three days following spraying, and were considered altogether since then. The merging of bands is a natural phenomenon in areas presenting high density of bands, explained by their considerable mobility. In some instances, 2<sup>nd</sup> instar nymphs were recorded moving as fast as 100 m a day.

Population reduction of *R. schistocercoides* nymphs at the end of the experiment ranged from 81.7 to 84.1% for the low dosage bands (L1 and L3) and from 61.6 to 79.6% for the high dosage bands (H1/H2 and H3). Interestingly, the light rain during application time of bands L1 and L2, and wet vegetation during spraying of bands L3 and H1, did not hamper the achievement of satisfactory control levels. In contrast to studies in which water-based formulations were used (Inglis et al., 1995), precipitation seems not to considerably reduce the attachment of oil-formulated conidia to hydrophobic surfaces, specially grasshopper cuticles and grass parts. An illustrative diagram of the structural changes in an untreated and treated bands is shown in Figure 1.

The values are close to the 88% reduction obtained in the 1998 field trial when a dosage equivalent to  $2.0 \times 10^{13}$  conidia/ha was applied (Magalhães et al., 2000b). The results clearly suggest that economically feasible dosages (a quarter of that used in 1998) have a significant impact on grasshopper populations. Different studies have shown the possibility of achieving high control levels under field conditions applying dosages as low as  $1.0 \times 10^{12}$  to  $3.0 \times 10^{12}$  conidia/ha (Hooper et al., 1995; Price et al., 1997). Worldwide, the use of fungi for regulation of grasshopper populations is a successful biological control strategy and reviews on this subject can be found elsewhere (Goettel, 1992; Lomer, 1997; Magalhães et al., 2000a). Reports claiming successful control of other pests using field rates lower than  $5.0 \times 10^{12}$  conidia/ha are rare, mainly in open field environments, moreover in semi-arid regions.

To date, it is not known if *R. schistocercoides* is able to thermoregulate (capability of controlling body temperature through active physiological or behavioral mechanisms), as observed for *Melanoplus sanguinipes* (Boorstein & Ewald, 1987), *Taeniopoda eques* (Whitman, 1988) and many other species (Chappell & Whitman, 1990). It is clear that during the rainy season, when the nymphal stage occurs and maximum temperatures are around 30°C, any increase in body temperature is not being able to deter the fungal infection. In fact, during the peak of the epizootics, usually from 11 to 14 days post-spraying, dead insects were found in stem grasses in a basking position.

**Table 3.** Percentage of mortality of the hopper *Rammatocerus schistocercoides* nymphs fed on native grass sprayed with *Metarhizium anisopliae* var. *acridum* ( $1.0 \times 10^{13}$  viable conidia/ha).

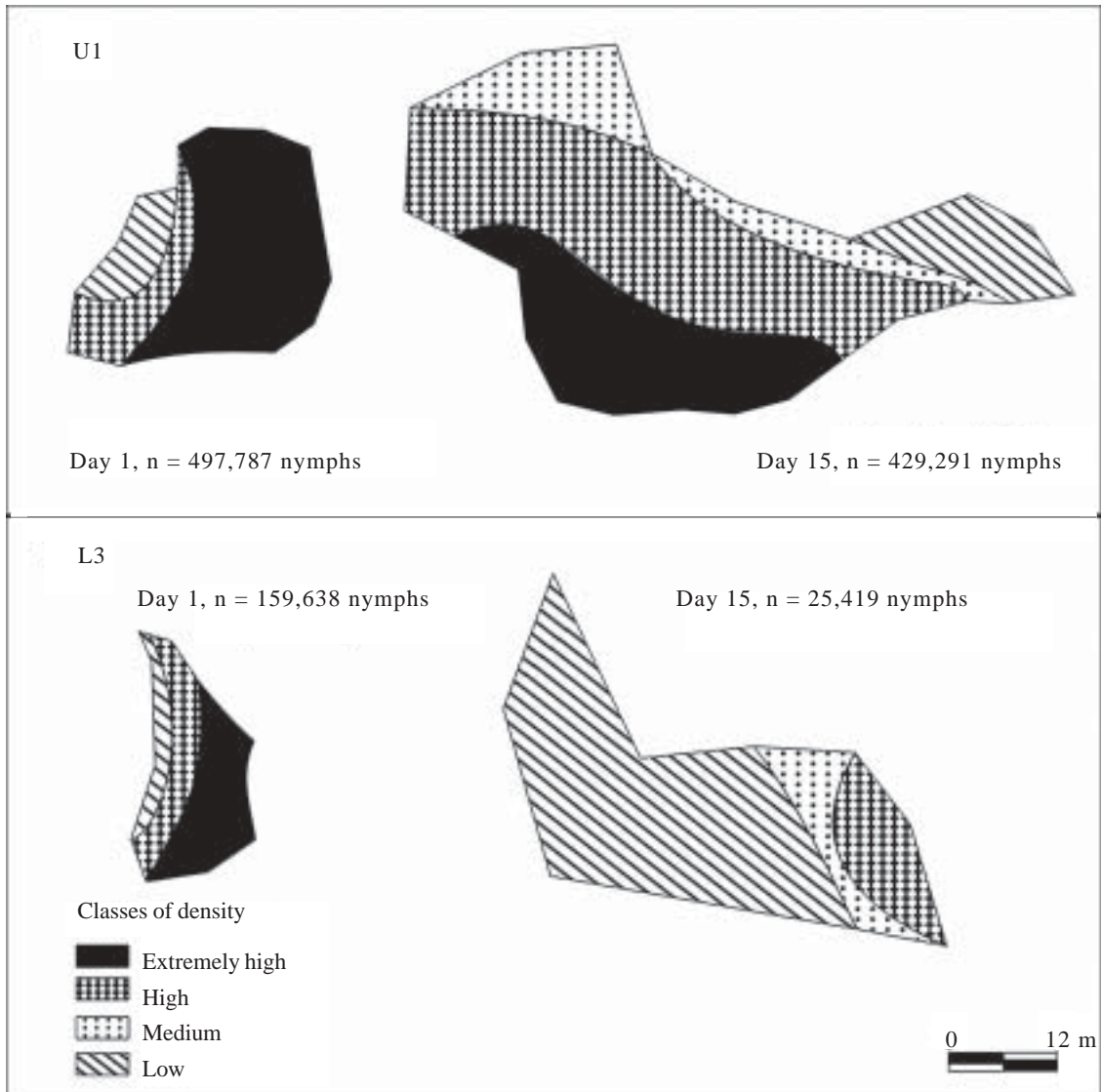
Replicate	Days after spraying					
	Total mortality			Confirmed mortality		
	Day 0 <sup>(1)</sup>	Day 2	Day 4	Day 0	Day 2	Day 4
1	63.3	96.7	70.0	43.3	46.7	10.0
2	57.9	77.8	36.7	36.8	38.9	20.0
3	-( <sup>2</sup> )	61.9	20.0	-( <sup>2</sup> )	19.0	10.0
Average <sup>(3)</sup>	60.6±1.90a	78.8±2.63a	42.2±6.19a	40.1±5.71a	34.9±4.35a	13.3±34.4a

<sup>(1)</sup>Grass leaves were collected immediately after application of the fungus and kept overnight at room temperature for 12 hours before offering to insects. <sup>(2)</sup>Data is missing (insects escaped from cage at day nine). <sup>(3)</sup>Within the same mortality parameter, average numbers±standard error followed by the same letter are not significantly different according to one way ANOVA.



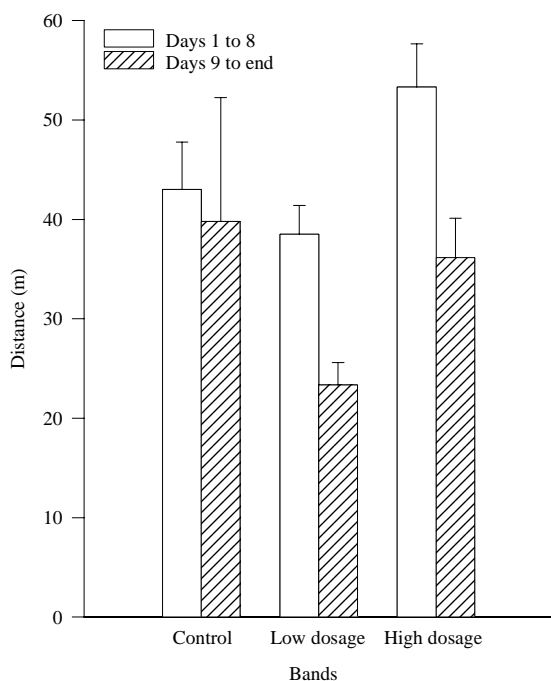
In the field trial the distances daily traveled by treated nymphs, when compared to untreated insects, were not severely affected. During the first eight days, average distances daily traveled in the control, and treatments with  $5.0 \times 10^{12}$  and  $1.0 \times 10^{13}$  conidia/ha were  $43.0 \pm 4.77$ ,  $38.5 \pm 2.90$  and  $53.3 \pm 16.75$  m, respectively (Figure 2). From eight days post-spraying to the end of the experiment, nymphs from the

control moved 41.7 m a day, on average. In contrast, treated nymphs reduced the traveled distance to 29.0 and 38.8 m a day, respectively. Studies carried out under laboratory conditions have demonstrated that sixth instar *R. schistocercoides* nymphs topically inoculated with 5,000 conidia of *M. anisopliae* var. *acridum* (CG 423) presented a significant reduction in food intake from day three onward (Faria et al.,



**Figure 1.** Illustrative diagrams of untreated (band U1) and fungus-treated (L3) bands of *Rhammatocerus schistocercoides* at the beginning (day 1) and end (day 15) of the field trial.

1999). The authors also observed that treated insects were less mobile than healthy ones and usually succumbed to fungal infection before the 10<sup>th</sup> day. There are some reasons that may explain the relatively long distances moved by treated nymphs under field conditions: conidial dose effectively delivered to each nymph is much lower than 5,000; and, nymphs were always collected in the front line of the bands, characterized for the high density of fast moving grasshoppers. The sampling procedure was biased toward fast moving nymphs, the ones that were less affected by the fungus. In order to solve this doubt, nymphs were collected 11 days post-spraying within spots of band H1/H2 with extremely high (front line), high, medium and low nymphal density (Table 4). Mortality rates observed in medium and low density spots were considerably higher, corroborating the hypothesis that sick nymphs, incapable of following healthy ones, tend to be found in low density zones (the ones located in the back of the band).



**Figure 2.** Average distances daily traveled by bands of grasshopper nymphs, *Rhammatocerus schistocercoides*, treated with low ( $5.0 \times 10^{12}$ ) and high ( $1.0 \times 10^{13}$  conidia/ha) dosage of fungus *Metarhizium anisopliae* var. *acridum*.

**Table 4.** Percent mortality rates of the hopper *Rhammatocerus schistocercoides* nymphs collected in different density zones within a treated band at 11 days post-spraying with fungus *Metarhizium anisopliae* var. *acridum* and caged for seven days.

Mortality (%)	Density zone <sup>(1)</sup>			
	Extremely high	High	Medium	Low
Total	22.0	24.0	95.0	84.1
Confirmed	2.0	4.0	30.0	27.3

<sup>(1)</sup>Densities zones extremely high, high, medium and low correspond to 2,512, 696, 192 and 39 nymphs per m<sup>2</sup>, respectively.

In fact, in the second half of the trial it was common to find groups of sluggish nymphs, not responsive to humans approximation. It was possible to observe a high number of spiders, ants and *Calosoma* sp. (Coleoptera: Carabidae) preying on *R. schistocercoides* nymphs. In the last days there were hundreds of reddish dead bodies on tracks of the treated bands, whereas this did not happen in the control treatment.

This study provides additional evidences for use of *M. anisopliae* var. *acridum* as mycoinsecticide for grasshopper control. Field trials to test other dosages and the impact of the fungus on non-target organisms are under way.

## Conclusions

1. Nymphs of *R. schistocercoides* are susceptible to field dosages of *M. anisopliae* var. *acridum* as low as  $5.0 \times 10^{12}$  conidia/ha.
2. Mortality rates for nymphs collected up to two or four days post-spraying are not affected when the dosages  $5.0 \times 10^{12}$  conidia/ha and  $1.0 \times 10^{13}$  conidia/ha are applied.
3. High mortality rates for nymphs fed to grasses treated with *M. anisopliae* var. *acridum*, up to four days post-treatment, reinforce the argument that persistency of the fungus in the field is not severely affected by adverse environmental conditions at Campos de Júlio, MT.

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