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Full Length Research Paper

Toxicity of the insect growth regulator lufenuron on the entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin assessed by conidia germination speed parameter

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***Metarhizium anisopliae* has been considered a promising alternative with low environmental impacts for the biological control of a variety of insect-pests. Another alternative is the use of biological pesticides such as insect growth regulators, including lufenuron. An assessment of the potential impact of fungicides on *M. anisopliae* is of critical importance to integrated pest management, permitting the compatible use of this entomopathogen with chemical defensives. Based on this, this study aimed to evaluate the effect of different concentrations of lufenuron on the conidia germination speed of *M. anisopliae*. Conidia were incubated at 28°C and sampled throughout 12 h. Bayesian analysis showed an inhibition of conidia germination in the presence of 2.0 mg/ml of lufenuron, whereas their compatibility was observed in the concentrations of 1.0 mg/ml and 700 µg/ml. It indicates that in these last two concentrations, the fungicide has no toxicity on *M. anisopliae*, what suggests that it can be employed in biological-chemical combinations, maintaining viable the fungal inoculums after its application in the field, with a low environmental impact.**

Key words: Entomopathogen, biological control, vegetative development, biological-chemical combinations.

INTRODUCTION

The last decades has been increasing the fact that humans are concurrently exposed to various chemicals agents via food and environment (Reffstrup et al., 2010), enlarging the risks of genetic toxicity, cancer, birth defects, kidney or liver disease (Mattsson, 2008). Also, these defensives can cause an increased predisposition to diseases or a temporary reduction in the plant and the soil properties; microorganisms and hosts can be also affected (Altman and Campbell, 1977).

Based on the controversy generated about the excessive use of chemical pesticides, more biological pesticides such as insect growth regulators (IGRs) have been applied to combat crops pests (Godfrey, 1995; Payá et al., 2009). IGRs present many advantages compared with conventional products because they are not much toxic to mammals or natural enemies (Dhadialla et al., 1998).

Lufenuron (RS)-1-[2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy) phenyl]-3-(2,6-difluorobenzoyl)urea) is known as an insect development inhibitor/ insect growth regulator. It is active against larval developmental stages, causing cuticular lesions and interfering in the chitin biosynthesis (Dean et al., 1998; Moriello et al., 2004).

It is principally used for controlling the cat flea, *Ctenocephalides felis* (Dean et al., 1998), being also active against diptera (Wilson and Cryan, 1997). It is also employed to control pests of several vegetal crops,

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including the citrus rust mite *Phyllocoptruta oleivora* (Bueno and Freitas, 2004) and adult predators of cotton, as earwings, ladybugs, spiders, mirids and green lacewings (Castane et al., 1996; Angeli and Forti, 1997; Javaid et al., 1999).

Another promising alternative with low environmental impact for biological control of insect-pests is the use of entomopathogenic fungi that are considered supplements or alternatives to synthetic insecticides (St. Leger et al., 1996). More than 150 insect biocontrol products based on fungal entomopathogens have been commercialized in the last years (Faria and Wraight, 2007).

Metarhizium anisopliae (Metschnikoff) Sorokin is an asexual filamentous fungus capable of infecting more than 300 species of insect-pests (Roberts and St. Leger, 2004). Based on it, this fungus is commercially produced by Australia, South Africa, Germany and Brazilian companies (Khetan, 2001; Scholte et al., 2004a). Its action has already been reported against several plant and animal plagues, such as: African tephritid fruit flies (Dimbi et al., 2004), spittlebugs on sugarcane and pastures (Isaka et al., 2005), ticks of Ixodidae family (Lübeck et al., 2008), black maize beetle (Makaka, 2008), sugarcane whitegrubs (Allsopp, 2010) and *Haematobia irritans* (Mochi et al., 2010). A recent study conducted by Niassy et al. (2011) showed *M. anisopliae* as a promising candidate for the biological control of locusts and grasshoppers.

Its potential for controlling human plagues is also reported. Several researches have related the potential of *M. anisopliae* to the biocontrol of adult African malaria insect-vectors, including *Anopheles gambiae*, *Anopheles funestus* and *Anopheles stephensi* (Scholte et al., 2003, 2004b, 2005; Farenhorst et al., 2008; Kannan et al., 2008). In Brazil, this fungus is being emphasized as a potential candidate for the biocontrol of *Aedes aegypti*, vector of dengue (De Paula et al., 2008; Luz et al., 2008) and triatomines, such as *Triatoma infestans*, which transmits *Trypanosoma cruzi*, the agent of Chagas disease in Latin America (Luz et al., 2004; Lazzarini et al., 2006).

The knowledge of the potential impact of fungicides on this fungus is of critical importance to the successful integration of this agent into integrated pest management, permitting the compatible use of this entomopathogen with chemical defensives. The compatibility analysis of commercially entomopathogenic fungi with soil-applied fungicides can be conducted *in vitro* by adding products to the synthetic culture media used for fungal growth (Mochi et al., 2005). Chitin is an important component of the exoskeleton of arthropods and is also found in the outer cell wall of fungi, not being found in mammalian cells. It is possible that some compounds that affect chitin synthesis may have antifungal activity without presenting a risk of host toxicity (Moriello et al., 2004).

Because of the importance of *M. anisopliae* as a microbial agent of a wide variety of insect-pests, it is important to value the effect of chemical defensives on this fungus, considering the conidia germination speed parameter, which is directly associated with virulence. Therefore, this study aimed to verify the effect of different concentrations of lufenuron on the conidia germination speed of Mato Grosso (MT) strain of *M. anisopliae*.

MATERIALS AND METHODS

Fungal strain and culture media

MT strain of *M. anisopliae* var. *anisopliae* was obtained from the fungal culture collection of Laboratório de Biotecnologia Microbiana from Universidade Estadual de Maringá, Paraná, Brazil. This fungus was isolated from the insect host *Deois* sp. Complete medium (CM) and liquid complete medium (LCM) (Pontecorvo et al., 1953) were employed.

Conidia germination speed in the presence of lufenuron

The MT strain was incubated in Petri dishes containing CM (20 ml) in biological oxygen demand (BOD) at 28°C. Conidia were obtained directly from seven days-old sporulating cultures by scraping and then suspending in aqueous solution of 0.01% Tween 80 (5 ml). The suspended conidia were inoculated into four Erlenmeyer flasks containing LCM (4 ml) in a concentration of 3.23×10^7 conidia/ml. One of them was used as the negative control (C); the three treatments received lufenron (Program®) in different concentrations: 700 µg/ml (T1), 1 mg/ml (T2) and 2 mg/ml (T3). All Erlenmeyer flasks were incubated in BOD at 28°C for 12 h. Samples from the control and each treatment were collected at 2, 4, 6, 8, 10 and 12 h of incubation, and then germinated conidia were counted using Neubauer hemocytometer.

The percentage germination and germination speed were assessed by randomly observing 300 conidia. A conidium was considered germinated when a germ-tube projected from it (Milner et al., 1991). Each treatment was replicated five times and the entire assay was performed thrice.

Statistical analysis

When the data has no normal distribution, one can use logarithmic transformations, but it could provide erroneous results once this methodology transforms the data and not the parameters in the model. One approach to overcome the problem of non-normalized distributions is generalized linear models that consider the likelihood distribution of the data, but still presents biases problems with small datasets. The Bayesian statistics is another approach that can work on datasets considering the true distribution and it is reliable for small groups of data.

To verify the possible differences in the number of germinated conidia among treatments, incubation times and their interactions data were analyzed using statistical package BRugs for software R (2008) and the Poisson distribution was assumed, implemented in Bayesian methodology. For each parameter, 10,000 values were generated in a *Monte CarloMarkov Chain* (MCMC*)* process, considering a sample discard period of 1,000 initial values. The final sample was taken with steps of 10, that is at every 10 values generated, one was taken to belong to the sample, with 900 values generated. The multiple comparisons procedure was based on a posteriori samples of the estimates of the parameters. Significant differences were considered at the level of 5% between the treatments if the zero value was not

contained in the credibility interval of the desired contrast. A non-informative Gamma distribution was considered a priori for means of germinated conidia, that is, $\theta_n \sim G(10^{-3};10^{-3})$, where θ_n is the mean for each *n* treatment considered.

To study the behavior of conidial germination over incubation time, for each treatment and control, a model of logistic regression was applied, and implemented in Bayesian methodology. Data were analyzed using statistical package BRugs for software R (2008) according to the formula:

$\log it(\theta ij) = \beta_0 + \beta_1 time + \beta_2 time^2$,for the control and treatment 1 and

$\log it(\theta ij) = \beta_0 + \beta_1 time$, for treatments 2 and 3

Where, log *it* is the logistic link function; θij is the germination percentage; β_0 is the intercept; β_1 is the linear logistic regression coefficient; β_2 is the quadratic logistic regression coefficient and *time* is the number of hours elapsed since the beginning of incubation. The regression fit was tested by the coefficient of determination (r^2). The binomial distribution was considered for the data of germination percentage.

For each parameter, 50,000 values were generated in a MCMC process, considering a sample discard period of 10,000 initial values. The final sample was taken with steps of 15, with 2,667 values generated. The significance of logistic regression coefficients was considered at the level of 5% if the zero value was not contained in the credibility interval for the parameter. A non-informative normal distribution was considered a priori for parameters b_0, b_1 and b_2, that is, $b_0, b_1, b_2 \sim N(0;10^{-6})$.

When a logistic link function is considered, the conidia germination percentage is generally given by:

$$\theta_{ij} = \frac{\exp(\beta_0 + \beta_1 time)}{1 + \exp(\beta_0 + \beta_1 time)}$$

When only the linear effect was significant, or

$$\theta_{ij} = \frac{\exp(\beta_0 + \beta_1 time + \beta_2 time^2)}{1 + \exp(\beta_0 + \beta_1 time + \beta_2 time^2)}$$

When the quadratic effect was also significant.

RESULTS AND DISCUSSION

The means and ICr for counting the germinated conidia are shown in Table 1. A Bayesian ICr of 95% is the interval in which 95% of the samples are contained, and smaller the interval, less dispersed is the parameter. The means of germinated conidia in 12 h were: 39.180 (C), 47.13 (T1), 35.19 (T2) and 9.512 (T3), with credibility interval formed by 2.5 and 97.5%.

Bayesian analysis showed that there was a significant difference between treatments 1 and 3, been the conidia germination in the concentration of 2 mg/ml (T3) lower than the germination with 700 µg/ml (T1) of lufenuron, which significantly increased the conidia germination, when compared with control.

The conidia germination is directly influenced by the incubation period, which is an important factor in the studies of entomopathogen resistance in the presence of a growth regulator. The means of conidia germination in each incubation period sampled throughout the 12 h and the credibility intervals can be seen in Table 2.

According to these results, the incubation periods of 2 and 4 h were statistically equal, showing a low germination percentage. This occurrence was not due to action of lufenuron, because the physiological evolution of conidia germination started to be apparent from 8 h of incubation. Also, the periods of 10 and 12 h were statistically equal, showing that the compatibility tests can be finished two hours sooner, without affecting the germinative behavior.

Observing the interaction analyses among incubation periods and treatments (Figure 1), it was possible to verify if, into each period, the conidia germination kept the same standard among the treatments. At 8 h (apparent germination period) and at 10 h (final germination period for this test) of incubation, the conidia germination speed in T1 was bigger than the one observed in C, indicating that 700 µg/ml did not affect negatively the MT conidia germination, otherwise, it increased conidia germination. At 8 h, a significant decrease in the germination speed was observed in T2 and T3, been the last one which presented the lowest germination activity.

According to the curve of germination speed, the conidia germination started near 6 h of incubation for C, T1 and T2, with the highest initial velocity in C, which decreased after 10 h, whereas the velocity increased in T1 and T2, the highest velocity observed in T1. In T3, the conidia germination started after 8 h of incubation and it increased with a linear and slow behavior. The observed decrease on conidia germination in the control was similar to a growth curve, a plateau (in which maximum germination occurs) which was expected.

The logistic regression adjusted efficiently the conidia germination percentage over time and the coefficients of determination (r^2) are shown in Table 3. The behaviors of C and T1 were similar, where the germination had a quadratic behavior, whereas in T2 and T3, it was linear.

Our results show that lufenuron was not capable of inhibiting the MT conidia germination when applied in the concentrations of 700 µg/ml and 1 mg/ml, because germination developed over time. The same was not observed in 2 mg/ml of lufenuron, when the germinative activity was statistically lower compared with the negative control and it presented a lower velocity. The effect of increasing the germination of conidia of *M. anisopliae* when the lufenuron was used at the concentration of 700 µg/ml and the opposite effect when used in higher concentration (2mg/mL), with inhibitory conidia germination effect, are an interesting data that could indicate the hormesis occurrence. According to Thong and Maibach (2008), hormesis, or biological effects of low level exposures (BELLE) in the field of toxicology, is characterized by non-monotonic dose response which is biphasic, displaying opposite effects at low and high doses. Its occurrence has been documented across a broad range of biological models and diverse type of exposure (Calabrese and Baldwin, 2001, 2003; Calabrese 2005a, b;

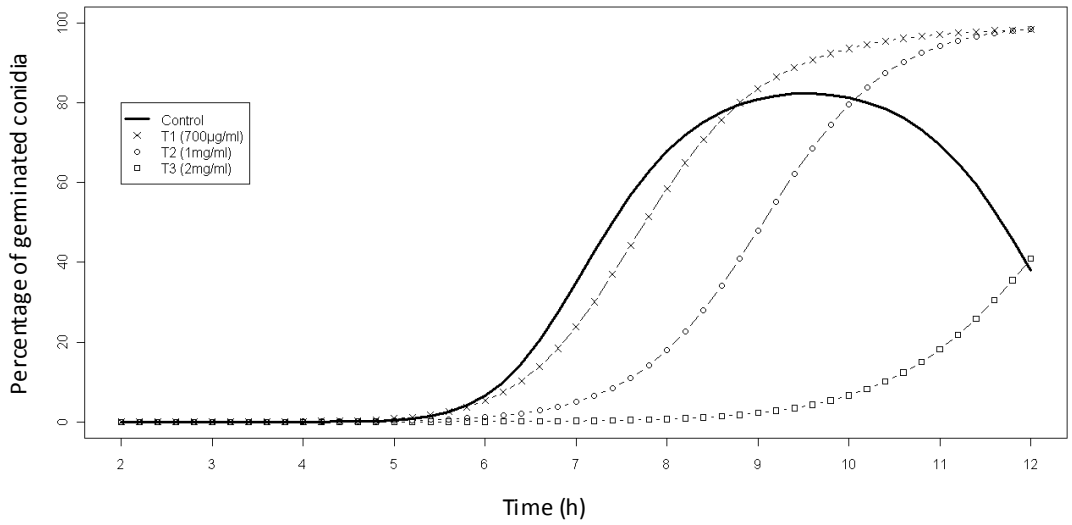

Figure 1. The curve of germination speed of *M.anisopliae* conidia in the control and treatments.

Table 1. Bayesian estimates for the counting of germinated *M. anisopliae* conidia in the presence of different concentrations of lufenuron.

Treatment	Mean	Standard error	95% ICr	
			2.50%	97.50%
Control (C)	39.180[b]	0.047	36.350	42.140
700 µg/ml (T1)	47.130[a]	0.042	44.080	50.160
1 mg/ml (T2)	35.190[b]	0.039	32.600	38.060
2 mg/ml (T3)	9.5120[c]	0.022	8.051	11.020

[(a,b,c)] Different letters indicate that the means differ.

Table 2. Means and credibility intervals for counting of the germinated *M. anisopliae* conidia throughout the incubation period.

Time (h)	Mean	Standard error	95% ICr	
			2.50%	97.50%
2	0.085[d]	0.086	0.003	0.306
4	0.252[d]	0.150	0.053	0.636
6	4.497[c]	0.614	3.395	5.828
8	39.160[b]	1.801	35.770	42.740
10	78.190[a]	2.560	73.480	83.350
12	74.250[a]	2.490	69.370	79.040

[(a, b, c, d)] Different letters indicate that the means differ.

Table 3. Bayesian estimates for the logistic regression coefficients for control and treatments.

Coefficient	b0	b1	b2	R^2
C	-29	6.404	-0.336	0.945
T1	-17.850	3.155	-0.110	0.997
T2	-13.030	1.439	-	0.995
T3	-14	1.136	-	0.972

b0 is the intercept; b1 is the linear coefficient; b2 is the quadratic coefficient and r^2 is the determination coefficient of regressions.

Brugman and Firmani, 2005; Thong and Maibach, 2008; Pereira et al., 2009). The low-dose stimulatory response often occurs following an initial disruption in homeostasis and appears to represent a modest overcompensation response. It is believed that the modest stimulatory responsiveness is due to the result of a compensatory process that "slightly" overshoots its goal of the original physiological set-point, ensuring that the system returns to homeostasis without unnecessary and excessive overcompensation (Calabrese, 2001). Therefore, it is important to follow the dose-response relationships overtime in order to better define its quantitative features (Thong and Maibach, 2008).

The dose-response test in relation to the observation of different incubation times was employed with success in this study with lufenuron and *M. anisopliae*. The differences in germination taxa and velocity, observed in *M. anisopliae* incubated with lufenuron in different doses, compared with the control, could be explained, as related by Calabrese (2005b), by a existing molecular tactic that involves the presence of two receptor subtypes affecting cell regulation, one with high and other with low affinity for agonist but with notably more capacity (that is, more receptors). Such an arrangement may lead to the biphasic dose response, with the high-affinity receptor activated at low concentrations, which stimulates DNA synthesis and cellular proliferation; and the low affinity/high-capacity receptor becoming dominant at higher concentrations decreasing the cell proliferative response (Thong and Maibach, 2008).

Lufenuron is used for controlling fungal infections in several animal species, due to its capacity to tear chitin from fungal cell wall, as well as, to inhibit the synthesis, polymerization and deposition of chitin. However, analysis of variance and Fischer's least significant difference test, made by Scotty et al. (2005), indicated that it showed no effect on the *in vitro* growth of *Aspergillus* spp. and *Fusarium* spp. Also according to them, the chitin synthesis inhibition could fail in filamentous fungi, due to the absence of peptide transporters (enzymes) that load the synthesis inhibitors from cell wall as far as the active site of chitin synthesis.

The same authors emphasized that the higher used concentration of lufenuron (700 µg/ml) was the highest that could be tested *in vitro*, because of its opacity formulation, as well as the dilution effect resulted from the combination of chemical agent with the fungal suspension. In our study, 700 µg/ml was our lowest concentration tested (T1) and, in agreement with Scotty et al. (2005), we also did not observe the inhibition of the conidia germination. Hector et al. (2005) evaluated and compared the *in vitro* antifungal properties of lufenuron against isolates of *Coccidioides immitis* and *Aspergillus fumigates*, used singly and in combination with the azole antifungal agent itraconazole. No evidence of inhibition, either by susceptibility testing or direct microscopic examination of treated cells, was obtained with lufenuron under experimental conditions. On the basis of their *in vitro* data, Hector et al. (2005) concluded that lufenuron did not appear to possess antifungal properties.

Differently, Purwar and Sachan (2006) performed investigations to study the effect of *M. anisopliae* on the toxicity of lufenuron, among other chemical insecticides, against 10 to 11 days old larvae of *Spilarctia obliqua*, and showed no difference in toxicity against *S. obliqua.* The authors considered that this result may be due to the death of spores of *M. anisopliae* due to this insecticide.

The conidia germination speed parameter was also applied by Rangel et al. (2004) to verify the influence of growth substrate and nutritional environment on the conidial UV-B tolerance of two isolates of *M. anisopliae* var. *anisopliae.* As a result, conidia from insect cadavers germinated slower than those from PDAY culture medium. Rangel et al. (2005) made another similar study with two strains of *M. anisopliae* var. *acridum*, observing that conidia produced on artificial or natural substrates have a similar culturability and tolerance to UV-B radiation, but conidia from artificial substrate germinated faster and with a higher germination rate than conidia from natural substrate.

The effects of physical and nutritional stress conditions during mycelial growth were assessed by Rangel et al. (2008) using different parameters, including the conidial germination speed. According to them, when compared with control conidia, the ones generated under nutritive stress increased their germination speed, whereas conidia produced on media with high osmolarity showed a faster germination and those produced from mycelium UV-A irradiated had no increased germination rate.

Similarly, the conidia germination speed, among other parameters, was also employed by Safavi et al. (2007) to evaluate the effect of nutrition on virulence of *Beauveria bassiana* and *M. anisopliae*. Seven different media culture were used for fungal growth and *B. bassiana* conidia grown on "osmotic stress" medium, presented the highest germination rate, while for *M. anisopliae* conidia, the high-C/N medium induced the highest germination rate.

The mentioned studies showed that germination rate is a pathogenicity determinant that is affected by nutritional conditions. In agreement with these, t he results revealed that culture media supplemented with lufenuron influence the conidia germination of *M. anisopliae* strain MT.

Based on our results, lufenuron was not interfered in the MT conidia germination when used in a concentration of 1 mg/ml and increased it in a concentration of 700 µg/ml. This compatibility between the IGR and *M. anisopliae* indicates that it is not toxic to the entomopathogenic fungus and suggests that they can be mixed and used in a biological-chemical combination to combat insect-pests, maintaining the inoculum source (conidia) in the field after application.

Considering the foregoing, the integrated use of lufenuron and *M. anisopliae* in pest management could be proposed to the future, with the objective of testing the possibility of controlling the insects plagues of *Spodoptera frugiperda* and *Alabama argillaceae* by this chemical pesticide and the entomopathogen together. In Brazil, *S. frugiperda* (J. E. Smith) is a common pest specie in corn plantations. It is a pest of great economic importance (Beserra et al., 2002).

The cotton leafworm, *A.argillacea* (Huebner, 1818) (Lepidoptera: Noctuidae), is considered to be one of the key pests in herbaceous cotton (*Gossypium hirsutum* L. r. *latifolium* Hutch) cropping, with constant occurrence in all cotton-growing states of Brazil (César Filho et al., 2002). The lufenuron pesticide was proved to be efficient against *S. frugiperda* (Cruz et al., 2010) and *A. argillaceae* (Scarpellini, 2001). In the same way, *M. anisopliae* have a potential as a microbial control agent against *S. frugiperda* (Lezama-Gutierrez et al., 1996; Lecuona and Díaz, 2001) and *A. argillaceae* (César Filho et al., 2002).

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