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Compatibility of entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin with fungicide thiophanate-methyl assessed by germination speed parameter

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Abstract

The biocontrol agent *Metarhizium anisopliae* is efficient to combat more than three hundred species of insect pests and can be used in biological-chemical combinations with chemical defensives maintaining the inoculum source of fungi in the field. Studies of conidiogenesis in *M. anisopliae* are fundamental, considering that conidia are the main form of inoculum for biological control. Among the pesticides applied in pastures for pest control, to prevent and control plant diseases, is thiophanate-methyl. Due to the importance of *M. anisopliae* as a microbial agent of a wide variety of insect pests, it is of critical importance to evaluate the effect of chemical products 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 thiophanate-methyl on the conidia germination speed of MT (Mato Grosso) strain of *M. anisopliae*. Conidia were incubated with thiophanate-methyl in concentrations of 200 µg/ml (T1), 20 µg/ml (T2), 2 µg/ml (T3) and 0.2 µg/ml (T4) at 28ºC and sampled throughout 24 hours. The control was performed without the pesticide. Bayesian analysis showed an inhibition of conidia germination in the presence of 200 and 20 µg/ml of thiophanate-methyl. The curve of conidia germination speed showed that until 14 h of incubation, there was an increase in the germination speed of control and all treatments. Shortly afterwards, this speed decreased in T2 and T3 but it remained stable for C and T4. A stronger inhibition of conidia germination was caused by T1. The compatibility observed in concentrations of 20 and 2 µg/ml indicates that this fungicide could be mixed with *M. anisopliae* in biological-chemical combinations, maintaining the viable fungal inoculum after its application. To confirm it, third-instar larvae of sugarcane borer (*Diatraea saccharalis*) were infected with a combination of a conidia solution of MT strain and thiophanate-methyl in concentration of 20 µg/ml. As controls, water, conidia solution and the fungicide were applied separately. Food was offered *ad libitum* and larvae were monitored daily throughout 7-12 days at 25ºC and the factors of living larvae, larvae in pupal stage and dead larvae were evaluated. As results, the thiophanate-methyl did not affect the *D. saccharalis* larvae, but when these larvae were treated with *M. anisopliae* conidia mixed with the pesticide, it was observed a reduction of larvae mortality of 26.8% when compared with the use of *M. anisopliae* only (without pesticide).

Key words: Vegetative development, biological-chemical combinations, entomopathogen, conidia, Bayesian analysis, biological control, sugarcane borer, pupal stage, mortality.

Introduction

The increasing use of chemical products in order to implant and maintain healthy crops and high productivity has been causing negative effects for the biotic complex of nature, affecting animals, humans and plants [1]. Pesticides are held responsible for contributing to the loss of biodiversity [2,3], causing the pollution of air [4], soil [5] and surface water [6], increasing risks to the environment and human healthy [7].

Recently, Yarpuz-Bozdogan [8] reported that human health has been affected higher than environment in herbicide applications. This author also stated that some factors such as application doses, techniques and equipment and meteorological conditions should be taken into account for protection of human health and environment.

Biological control of pest insects has importance to the ecosystem for maintaining the balance of their population with a lower environmental impact [9]. An integrated management can combine chemical products and entomopathogens, being efficient for cultures that necessitate the use of chemical products [1,10]. Thus, determining the compatibility of commercially entomopathogenic fungi with soil-applied pesticides is of critical importance [11] and this has been conducted *in vitro* by adding products to the synthetic culture media used for fungal growth [1].

More than 150 insect biocontrol products based on fungal entomopathogens as *Metarhizium anisopliae*, *Beauveria bassiana*, *Beauveria brongniartii* and *Isaria fumosorosea* have been commercialized [12].

Metarhizium anisopliae var. *anisopliae* (Metschnikoff) Sorokin (order Moniliales, family Moniliaceae) is an asexual filamentous fungus. Its first use as a microbial control agent of insects was made by Elie Metschnikoff, in 1879, to control the wheat grain beetle (*Anisoplia austriaca*), afterward, it was used to control the sugar beet curculio (*Cleonus punctiventris*) [9], and in the past years several products based on *Metarhizium* spp. strains have been developed [13] for application in biological control and in the production of drug substances such as antibiotics and immunomodulators [14].

Because of its entomopathogenic characteristics, *M. anisopliae* is efficient to combat more than three hundred insect-pests [8]. In Brazil, it is employed to control spittlebugs on sugarcane and pastures [15]. In several countries, studies have been shown its potential to control a variety of human, animal and plant plagues, among them, African tephritid fruit flies [16], *Triatoma infestans* [17], *Haematobia irritan* [18] and sugarcane whitegrubs [19].

According to several characteristics, as the spore production, exoenzyme production, pathogenicity, resistance to chemical and physical agents and genetic diversity has been observed as natural variability in *M. anisopliae* [20] strains.

Studies of the conidial production in *M. anisopliae* are fundamental, considering that conidia are the main form of inoculum for biological control of pests. Environmental factors interfere in a particular way at each stage (vegetative growth and sporulation) of the life cycle of this fungus, what justifies the necessity of further studies about the genetic and environmental mechanisms that control its development [21].

Recent studies demonstrated that some isolates of *M. anisopliae*, when grown in liquid culture fermentation, differentiated to form sclerotial propagules [22], which were desiccation tolerant and germinated sporogenically in soil to produce conidia *in situ* that infected and killed susceptible soil dwelling insects. The sclerotia-containing granules showed more efficiency than granules made from conidia of *M. anisopliae* bound to a solid nutritive carrier [23].

The systemic broad-spectrum fungicide thiophanate-methyl (dimethyl 4,4'-(o-phenylene)bis(3-thioallophanate)) is a thioallophanate compound, belonging to the chemical group ftalonitrilas (chlorothalonil). It is used in pastures for pest control, to prevent and control plant diseases caused by various fungi and it presents a range of activity in comparison with other common fungicides as well as lower general toxicity [24].

Due to the importance of *M. anisopliae* as a microbial agent of a wide variety of insect pests, it is of critical importance to evaluate the effect of chemical products on this fungus, considering the conidia germination speed parameter, which is directly associated with virulence. An understanding of the potential impact of fungicides on this fungus is important to the successful integration of this agent into integrated pest management, permitting the compatible use of this entomopathogen with chemical defensives.

Therefore, this study aimed to verify the compatibility of entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin with fungicide thiophanate-methyl assessed by germination speed parameter and infection of *Diatraea saccharalis* Fabricius larvae.

Materials and Methods

Fungal strain, insect larvae and culture media: MT (State of Mato Grosso, Brazil) strain of *M. anisopliae* var. *anisopliae* was isolated from the insect host *Deois* sp. and belongs to the fungal culture collection of the Laboratório de Biotecnologia Microbiana from Universidade Estadual de Maringá, Paraná, Brazil. Third-instar larvae of sugarcane borer (*Diatraea saccharalis*) were provided by the Laboratório de Morfologia e Citogenética de Insetos from Universidade Estadual de Maringá. Complete Medium (CM) and Liquid Complete Medium (LCM) [25] were employed.

Determination of conidia germination speed in the presence of thiophanate-methyl: In Petri dishes containing CM (20 ml), the MT strain was incubated in biological oxygen demand (BOD) at 28ºC. Conidia were obtained directly from 7 days-old sporulating cultures by scraping and then suspending in 0.01% aqueous solution of Tween 80 (4 ml). Into five Erlenmeyer flasks containing LCM (10 ml) were inoculated the suspended conidia in a concentration of 3.28×10^7 conidia/ml. One of them was used as negative control (C), the four treatments received thiophanate-methyl in different concentrations: 200 µg/ml (T1), 20 µg/ml (T2), 2 µg/ml (T3) and 0.2 µg/ml (T4). All Erlenmeyer flasks were incubated in BOD at 28ºC for 24 h. Samples from control and each treatment were collected at 6, 8, 10, 12 and 24 h of incubation, 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 [26]. Each treatment was replicated five times and the entire assay was performed thrice.

Determination of compatibility of M. anisopliae and thiophanate-methyl during the infection of D. saccharalis larvae: In Petri dishes containing CM (20 ml), the MT strain was incubated in BOD at 28º C. Conidia were obtained directly from 7 days-old sporulating cultures by scraping and then suspending in 0.01% aqueous solution of Tween 80, generating a solution with a concentration of 9.30×10^6 conidia/ml.

Disks of sterile filter paper were placed on Petri dishes (9 cm). As controls, 300 µl of autoclaved distilled water (C1), 300 µl of conidia solution (9.30×10^6 conidia/ml) of MT (C2) or thiophanate-methyl in a concentration of 20 µg/ml (C3) were dripped on disks or were dripped on each filter paper disk. As treatment, 300 µl of a mixture of conidia solution of MT and thiophanate-methyl (2000 µl of 9.30×10^6 conidia/ml + 80 µl of thiophanate-methyl in a concentration of 20 µg/ml + 1920 µl of autoclaved distilled water) were dripped on filter paper disks.

Four larvae of *D. saccharalis* were placed on each Petri dish. Tests were made in at least four replicates for controls and ten replicates for treatment. Food (artificial diet) was offered *ad libitum* and dishes were incubated in BOD at 25ºC throughout 7-12 days and observed daily. The following factors were evaluated: living larvae, larvae in pupal stage and dead larvae.

Statistical analysis: Considering the conidia germination speed in the presence of thiophanate-methyl, to study the behavior of conidia germination over time, a model of logistic regression was applied, implemented in Bayesian methodology. Data were analyzed using statistical package BRugs for software R (version 2.7.0) [27] according to the formula:

$$\text{logit}(\theta) = \beta_0 + \beta_1 \text{time} + \beta_2 \text{time}^2$$

where logit is the logistic link function; θ 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.

It was also checked for possible differences in the number of

germinated conidia between treatments and control. The Poisson distribution was assumed. 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 contrast desired.

Non informative prioris were used for the parameters. There were 50,000 values generated in the iterative process, considering a sample discard period of 5,000 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 4,500 values generated. The convergence of the chains was verified by the CODA program [28] and by Heidelberger & Welch criteria [29].

Results and Discussion

The means and credibility interval (ICr) for counting of 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 is the interval, less dispersed is the parameter. The means of germinated conidia in 24 h were: 39.410% (C), 0.145% (T1), 17.700% (T2), 35.010% (T3) and 38.860% (T4), with credibility interval formed by percents 2.5 and 97.5%.

Table 1. Bayesian estimates for the counting of germinated *Metarhizium anisopliae* conidia in the presence of different concentrations of thiophanate-methyl.

Concentration	Mean	Standard error	95% ICr	
			2.50%	97.50%
Control	39.410[a]	2.390	34.880	44.250
200 µg/ml (T1)	0.145[c]	0.146	0.004	0.548
20 µg/ml (T2)	17.700[b]	1.577	14.700	20.930
2 µg/ml (T3)	35.010[a]	2.253	30.770	39.590
0.2 µg/ml (T4)	38.860[a]	2.368	34.320	43.620

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

According to these results, the germination of MT conidia was inhibited by the fungicide in T1 and T2, where a stronger inhibition was caused by T1. Both T3 and T4 did not inhibit the germination. The Bayesian analysis proved that there was no statistically significant difference between these treatments, in comparison with negative control.

According to Alves *et al*.[30], the classical statistical theory, regarded as a standard of analysis is often regarded as limited by assuming a normal distribution for the data when they behave not normal and do not provide accurate estimates when the sample size is relatively insufficient. In order to obtain estimates increasingly accurate, taking into account the distribution of data, the Bayesian analysis has been increasingly used.

The logistic regression adjusted effectively the percentage of germinated conidia over time, with average $r^2 = 0.895$. The values of regression coefficients for each treatment, with their respective r^2 can be seen in Table 2. The germination percentage over time showed a quadratic behavior.

Conidia germination is directly influenced by the time of incubation, which is an important factor for studying the resistance of entomopathogenic fungi in the presence of a chemical defensive.

Analyzing the curve of conidia germination speed (Fig. 1), we observed that until 14 h of incubation, there was an increase in the germination speed of control and all treatments. Shortly afterwards, this speed decreased in T2 and T3 but it remained stable for C and T4. The curve of T1 is not shown due to the occurrence of a germination inhibition of conidia by T1.

Table 2. Bayesian estimates for the logistic regression coefficients for control and treatments.

	b0	b1	b2	r^2
Control	-1.110	1.661	-4.726	0.907
T2	-1.263	1.704	-5.157	0.865
T3	-8.285	1.301	-0.040	0.858
T4	-7.601	1.048	-0.026	0.948

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

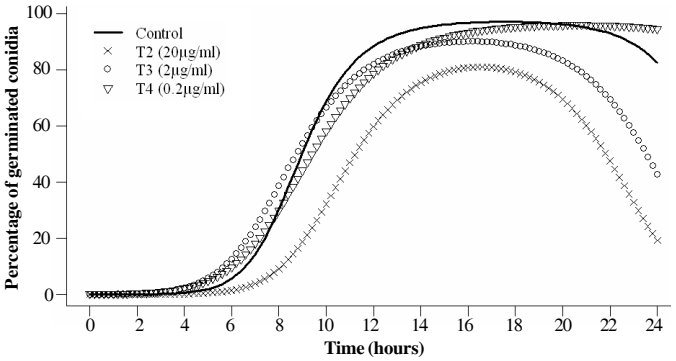

Figure 1. The curve of germination speed of *Metarhizium anisopliae* conidia in the control and treatments.

In order to evaluate the compatibility of *M. anisopliae* and thiophanate-methyl during the infection of *D. saccharalis* larvae, the factors living larvae, larvae in pupal stage and dead larvae were evaluated. The results are shown in Table 3.

Table 3. Percentage of *D. saccharalis* mortality and larvae in pupal stage treated with *M. anisopliae* conidia, thiophanate-methyl and the two combined.

Treatment	Living larvae	Larvae in pupal stage	Dead larvae
Water (C1)	75.00	25.00	0.00
MT conidia (C2)	33.33	26.67	40.00
Thiophanate-methyl (C3)	73.33	26.67	0.00
Water +thiophanate-methyl + MT conidia (T)	55.26	31.58	13.16

Similarly, Rangel *et al*.[31] used the germination speed as parameter to verify the influence of growth substrate (natural or artificial) and nutritional environment in which conidia are produced on the conidial UV-B tolerance and germination speed of two isolates (ARSEF 23 and ARSEF 2575) of *M. anisopliae* var. *anisopliae*.

In another study, Rangel *et al*.[32] observed that conidia of ARSEF 324 isolate of *M. anisopliae* var. *acridum* produced on artificial (PDAY culture medium) or natural (insect cadavers) substrates presented a similar culturability and tolerance to UV-B radiation. However, conidia produced on PDAY germinated faster and with a higher germination rate than conidia from infected adult *Melanoplus sanguinipes*.

In addition, the same researchers [33] demonstrated that the germination speed, adhesion and virulence of *M. anisopliae* var. *anisopliae* isolate ARSEF 2575 can be strongly influenced by culture conditions (including stresses) during production of the conidia. Like this, we have detected a slight and a strong influence of culture medium supplemented with low and high doses of thiophanate-methyl, respectively.

Bruck [11] tested the compatibility of a commercial isolate (F52) of *M. anisopliae* with several fungicides. Among them, thiophanate-methyl in concentrations of 0.5 and 0.9 g/L significantly inhibited the germination relative to the untreated control, when there was considered only the number of germinated conidia, not considering the germination speed, differently of the parameter used in our study.

Recently, a similar study was conducted by Alves *et al.*[30] that evaluated the toxicity of an insect growth regulator on *M. ansopliae conidia*. The insect growth regulator (IGR) lufenuron not interfered in the conidia germination speed of MT strain when used in a concentration of 1 mg/ml and increased it in a concentration of 700 µg/ml. It indicates that the IGR is not toxic to the *M. anisopliae*, suggesting 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.

The joint action between another entomopathogenic fungus, *Beauveria bassiana*, and synthetic pyrethroids was tested [34] against third instar larvae of *Helicoverpa armigera* (Hubner). The results showed that the larval mortality was 2.67-16.99 and 7.17-26.00 percent more than the expected mortality at LC_{50} of *B. bassiana* alone and LC_{50} of pyrethroids alone, respectively, what reinforces the compatible use of entomopathogens and chemical products in biological control of insect pests.

Acevedo *et al.* [35] evaluated the effect of interactions between the nematode *Heterorhabditis bacteriophora* (isolate JPM4) and two isolates of the fungus *M. anisopliae* (LPP45 and LPP39) during the infection of *D. saccharalis*. When each isolate was used alone, differences in larval mortality were observed: JPM4 caused 100% larval mortality within 5 days, the same was caused by LPP45 within 7 days and LPP39 only caused 60% over a 9-day period. For infections using LPP45 together with JPM4, 100% mortality occurred within 6 days of infection. When LPP39 was inoculated together with JPM4, the shortest time to 100% mortality was 4 days. These authors concluded that the combination of two highly virulent pathogens (LPP45 and JPM4) did not result in the most rapid host death, whereas the combination of a highly virulent nematode with a moderately virulent fungus (LPP39 and JPM4) caused the larvae die the fastest.

Our results showed that thiophanate-methyl was able to inhibit the germination of *M. anisopliae* (in the concentration of 200 µg/ml) or delay it (in the concentration of 20 µg/ml), not interfering in the conidia germination when used at lower concentrations. The use of thiophanate-methyl in field, depending on crop, p. ex. soya, is approximately 390-790 g/ha. However, the compatibility of thiophanate-methyl with this entomopathogenic fungus, when used combined, against *D. saccharalis*, is not high, with the 26. 84% reduction of larvae mortality when compared with the use of *M. anisopliae* conidia only (without pesticide).

Conclusions

A breeding program will be necessary which is focusing to the obtaining of *M. anisopliae* mutants more resistants to thiophanate-methyl considering biological control and integrated pest management.

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