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Sensibility of the entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin to *Pelargonium sidoides* extract (EPs 7630®) assessed by conidia germination speed parameter

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***Pelargonium sidoides*, known as umckaloabo, is a plant originally from South Africa and its extract is used in popular medicine. The fungus *Metarhizium anisopliae*, an important entomopathogen used in biological control of pests, is also employed as model for the evaluation of toxicity and compatibility of different products, considering, among other parameters, conidia germination speed. Therefore, this study aimed to evaluate the sensibility of *M. anisopliae* var. *anisopliae* in the presence of the extract of *P. sidoides*, at a concentration of 20.625 and 2.0625 mg/ml. During incubation at 28°C, samples were collected at 0, 6, 8, 10, 12 and 24 h and analyzed by light microscopy, with observation of 300 conidia, in triplicate, for both treatments and negative controls (in a completely randomized design). This study shows that the two concentrations of *P. sidoides* extract tested are capable of delaying the *M. anisopliae* conidia germination speed, in comparison to the controls, although the germination frequency has been restored after about 12 h of conidia incubations, with did not lack conidia viability, indicating no toxicity.**

Key words: Model fungus, toxicity, vegetative development, viability.

INTRODUCTION

The plant *Pelargonium sidoides* DC (Geraniaceae family) is native to the coastal regions of South Africa (Van der Walt and Vorster, 1988) and its root extracts has been widely used for the treatment of diarrhea and dysentery (Loureiro et al., 2005). Its ethanolic root extract, named EPs 7630® (Umckaloabo®), had full marketing authorization by the German drug regulatory agency (Conrad et al., 2007). It has antimicrobial and immunoregulatory activities (Franceschini et al., 2001) and it has been reported as efficient to treat herpes virus, respiratory infections, respiratory viruses (Rangel et al., 2004; Schnitzler et al., 2008; Michaelis et al., 2011). Its chemical composition is primarily phenolic and polyphenolic compounds, proteins, minerals, coumarin derivatives and a number of miscellaneous uncommon metabolites (Schötz et al., 2008). The early studies with the asexual filamentous fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin were conducted by the Russian researcher Elie Metschnikoff,

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in 1879, for the control of a species of beetle (Faria, 2001), and its great potential for the biological control of a variety of insect pests was already demonstrated (Makaka, 2008; Allsopp, 2010; Mochi et al., 2010; Niassy et al., 2011). Moreover, formulations based on it are commercialized by companies of Australia, Brazil, Germany, South Africa and USA (Khetan, 2001; Scholte et al., 2004; St. Leger and Wang, 2010). This fungus has been employed as model for evaluating its tolerance / compatibility / toxicity to different factors and substances (as UV-B radiation, fungicides, insecticides, insect grown regulators, pesticides, medicament) using parameters such as morphology, radial and vegetative growth, conidia germination speed (Rangel et al., 2004, 2005; Alves et al., 2011; Akbar et al., 2012; Tonussi et al., 2012; Fabrice et al., 2013; Bulla et al., 2013). Conidia germination speed can be explored as parameter to determine whether physical and/or chemical variables in the substrate on conidia are produced, having influence on fungal development and conidiogenesis (Rangel et al., 2004).

Considering that the *P. sidoides* extracts are used with medicinal application and *M. anisopliae* cells can be used as a referential of toxicity level of chemical products, as for example insecticides and medicaments, this study aimed to verify the sensibility of two concentrations of EPs 7630® extract on the conidia germination speed of *M. anisopliae*, expecting to detect the influence of this plant extract in an eukaryotic cell with economic importance, as an alternative citotoxic analysis of medicinal compounds, complementing another studies, such as mutagenicity tests with *Salmonella typhimurium*.

MATERIALS AND METHODS

Fungal strain, plant extract and culture media

The strain of *M. anisopliae* var. *anisopliae*, isolated from the insect host *Deois* sp., was obtained from the fungal culture collection of Laboratório de Biotecnologia Microbiana from Universidade Estadual de Maringá, Paraná, Brazil. The EPs 7630® (ethanolic root extract of *P. sidoides*) was employed. The culture media Potato Dextrose Agar (PDA) (Smith and Onions, 1983), complete medium (CM) and liquid complete medium (LCM) (Pontecorvo et al., 1953 modified by Azevedo and Costa, 1973) were employed.

Conidia germination speed in the presence of EPs 7630®

The *M. anisopliae* conidia solution was prepared according to Alves et al. (2011), except that the concentration 5.35×10^7 conidia/ml was obtained and inoculated into four flasks containing LCM. One of them was used as the negative control (C1); other received ethanol P.A. that was also used as negative control (C2). The two treatments received EPs 7630® in concentrations of 20.625 mg/ml (T1) and 2.0625 mg/ml (T2). All flasks were incubated in BOD at 28°C for 24 h. Samples from controls and treatments were analyzed in triplicate (three samples from each flask were collected) at 0, 6, 8, 10, 12 and 24 h of incubation. Germinated conidia were counted using Neubauer hemocytometer with light microscopy and the percentage of dormant, embedded, bud and germinated conidia was assessed by randomly observation of 300 conidia.

Viability of *M. anisopliae* conidia treated with EPs 7630®

The process described earlier was repeated, except that the conidia solution was obtained in a concentration of 6.20×10^7 conidia/ml. Samples from controls and treatments were collected at 8 h of incubation, diluted in concentrations of 10^2, 10^3, 10^4 and incubated in triplicate in Petri dishes (9 cm) containing CM (20 ml). Dishes were incubated in BOD at 28°C for 72 h and the number of macroscopic colonies was counted.

Statistical analysis

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. Significant differences were considered at the level of 5% between the treatments if the zero value was not included in the credibility interval of the desired contrast. The Markov chain was composed of 10,000 samples, with a burn-in period of 1,000 initial values and thin interval equal to 10.

The estimates were based on posterior means of the remaining 900 samples. Non informative priors were used for the parameters. For germination percentage over incubation time, a binomial distribution was assumed. Therefore, logistic regressions were implemented in Bayesian methodology. Data were also analyzed using statistical package BRugs for software R (2008) according to the model:

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

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 goodness of fit was checked by the coefficient of determination (r^2).

For this analysis, the Markov chain was composed of 10,000 samples, with a burn-in period of 1,000 initial values and thin interval equal to 10. The estimates were based on posterior means of the remaining 900 samples. A credibility interval (CI) of 95% was considered. Non-informative priors were used for the parameters. When a logistic link function is considered, the percentage of conidia germination can be obtained as:

$$\theta_{ij} = \frac{\exp(\beta_0 + \beta_1 time)}{1 + \exp(\beta_0 + \beta_1 time)}$$, for linear regressions, or

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

for quadratic regressions.

To verify the possible differences in the viability of *M. anisopliae* conidia in controls and treatments with EPs 7630®, data were also analyzed using statistical package BRugs for software R (2008) and the Poisson distribution was assumed, implemented in Bayesian methodology, as explained earlier.

Table 1. Bayesian estimates for the counting of germinated *Metarhizium anisopliae* conidia in the presence of *P. sidoides* extracts.

Treatment	Mean	Standard error	95% ICr	
			2.50%	97.50%
C1 (culture medium)	186[a]	0.03082	179.7	192.3
C2 (ethanol)	168.5[b]	0.02786	162.6	174.5
T1 (20.625 mg/ml)	93.2[d]	0.02455	88.77	97.73
T2 (2.0625 mg/ml)	100.5[c]	0.0239	95.84	105.2

[abcd] Different letters indicate that the means differ.

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

Time (h)	Mean	Standard error	95% ICr	
			2.50%	97.50%
0	0.009[f]	2.933E-4	5.966E-18	0.081
6	29.770[e]	0.01677	26.840	32.940
8	100.500[d]	0.03386	94.860	106.300
10	154.400[c]	0.03572	147.400	161.400
12	233.300[b]	0.04492	224.800	242.300
24	297.600[a]	0.05318	288.000	307.200

[abcd] Different letters indicate that the means differ.

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

Treatment	b0	b1	b2	R^2
C1 (culture medium)	-6.846	0.916	-	0.991008
C2 (ethanol)	-4.931	0.7123	-0.0117	0.9681967
T1 (20.625 mg/ml)	-1.418e + 01	1.249e + 00	-	0.9949418
T2 (2.0625 mg/ml)	-9.716e + 00	8.697e - 01	-	0.9955921

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

RESULTS AND DISCUSSION

Conidia germination speed in the presence of EPs 7630®

The entomophatogen important fungal *M. anisopliae* was chosen as model system to evaluate the effects of EPs 7630®, since fungi are considered model systems for studies about fundamental cell biological questions because basic principles of many cellular processes are conserved between fungi and animals (Steinberg and Perez-Martin, 2008; Bulla et al., 2013). The means and 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 186 (C1), 168.5 (C2), 93.2 (T1) and 100.5 (T2), with credibility interval formed by 2.5 and 97.5%. Bayesian analysis showed that there was a significant difference between treatments 1 and 2, being the conidia germination in the concentration of 20.625 mg/ml (T1) lower than germination with 2.0625 mg/ml (T2) of *P. sidoides* extracts. The two treatments significantly decreased the conidia germination speed, when compared with controls 1 and 2. According to Alves et al. (2011), the Bayesian statistical method is an approach that can work on datasets considering the true distribution, being reliable for small groups of data. The means of conidia germination in each incubation period sampled throughout 24 h and the credibility intervals are shown in Table 2. According to these results, the incubation periods of 0, 6 and 8 showed a low germination percentage of conidia incubated with *P. sidoides* extracts. Alves et al. (2011) observed that the physiological evolution of conidia germination started to be apparent from 8 h of incubation. In agreement with it, in the present study was observed that the conidia germination increased among 8 and 24 h of incubation. 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 C1, T1 and T2

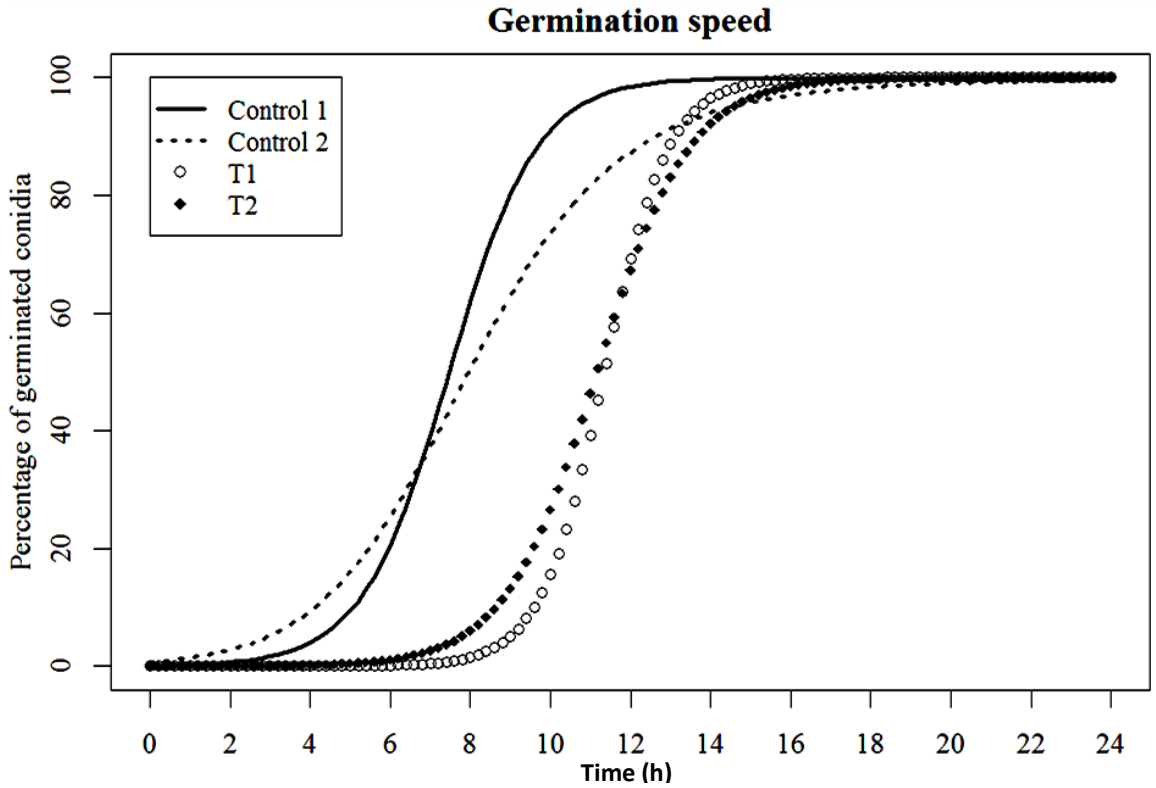

Figure 1. The curve of germination speed of *Metarhizium anisopliae* conidia in the control and treatments.

Table 4. Viability of *M. anisopliae* conidia in controls and treatments with EPs 7630® verified by the number of macroscopic colonies observed after 8 h of incubation. Means and credibility intervals for number of colonies were determined by Bayesian methodology.

Treatment	Mean	Standard error	95% ICr	
			2.50%	97.50%
C1 (culture medium)	2667.0[d]	0.9531	2606.0	2721.0
C2 (ethanol)	123330.0[a]	2.291	12210.0	12460.0
T1 (20.625 mg/ml)	5664.0[c]	1.214	5568.0	5750.0
T2 (2.0625 mg/ml)	6666.0[b]	1.344	6573.0	6758.0

[abcd] Different letters indicate that the means differ.

were similar, where the germination has a linear behavior, whereas in T2 and it was quadratic. The curve of germination speed (Figure 1) showed that the conidia germination started between 0 and 2 h of incubation for C1 and between 2 and 4 h of incubation for C2. The faster initial velocity was observed for C2, but the next 6 h of incubation, conidia in C1 has increased the speed of germination, passing C2. For T2, the conidia germination started near to 6 h of incubation, and it started near to 8 h of incubation for T1. Approximately after 16 h of incubation the germination speed of conidia remained stable for all controls and treatments.

The curve of *M. anisopliae* conidia germination speed obtained by Fabrice et al. (2013) showed that until 14 h of incubation, the germination speed increased for the control and all treatments of thiophanate-methyl (200, 20, 2 and 0.2 µg/ml); however, through incubation time this speed decreased and only it remained stable for controls and treatment with 0.2 µg/ml. Bulla et al. (2013) evaluating toxicity of the anti-hipertensive agent perindopril on the entomathogenic fungus *M. anisopliae* assessed by conidia germination speed parameter observed that the medicament concentrations of 200 and 20 µg/ml increased the germination speed of fungus conidia, indicating no toxicity. The results presented herein indicate that the two concentrations of *P. sidoides* extract tested are capable of delaying the *M. anisopliae* conidia germination, in comparison to the controls. All the germinated conidia has viability preserved (Table 4), that is, the capability of germinated conidia developing in a fungal colony. The process of spore germination can be defined as a sequence of events that activates the resting spore

(d'Enfert, 1997), what involves water uptake and wall growth (Griffin, 1994). The resting spore is converted into a rapidly growing germ-tube from which the mycelium will be formed by elongation and branching (d'Enfert, 1997). This process is directly influenced by the incubation period (Alves et al., 2011) and by environmental factors. Water, oxygen, and carbon dioxide are universally required to activate the spore germination (d'Enfert, 1997). More-over, optimum conditions such as temperature, humidity, pH and nutrient sources are essential for the conidia germination.

Germination speed of *M. anisopliae* conidia was similarly employed by Alves et al. (2011) as parameter to evaluate the toxicity of the insect grown regulator lufenuron. Bayesian analysis showed that conidia germination was not inhibited by the regulator in the lowest concentrations tested (700 µg/ml and 1 mg/ml). These authors suggested the possibility of a future use of a biological-chemical combination with *M. anisopliae* and lufenuron, with a low environmental impact to combat insect-pests.

Akbar et al. (2012) evaluated the toxicity of insecticides and fungicides on spore production and mycelial growth of *M. anisopliae*, showing that the chemicals composed by chlorpyrifos, phosphorus, metalaxyl + mancozeb and profenofos were the most toxic to mycelial growth and conidial germination. Meanwhile, the chemicals that contain acetameprid, cypermethrin, emamectin, imidacloprid and sinophos were less toxic to mycelial growth and spore production. Spinosad and indoxacarb were considered safe and compatible with *M. anisopliae*. The same methodology employed here was used by Tonussi et al. (2012) to evaluate the toxicity of deltamethrin on *M. anisopliae*. The authors observed that the concentration of 50 µg/ml reduced and delayed conidia germination and it was 100% inhibited by high concentrations. Ultra diluted treatments were not inhibitory with concentration of 31.25 ρg/ml and also with the concentration of 31.25 ηg/ml increased the germination of conidia, indicating a possibly occurrence of hormesis effect.

A recent study conducted by Fabrice et al. (2013) showed the efficiency of germination speed parameter to evaluate the compatibility of *M. anisopliae* Sorokin with fungicide thiophanate-methyl.

Data demonstrated that this compound inhibited or delayed the conidia germination, but not interfered in it when used at lower concentrations. The combined use of the entomopathogen and fungicide against *Diatraea saccharalis* larvae showed to be not high compatibly, since there was a 26.84% reduction of larvae mortality when compared with the use of *M. anisopliae* conidia only. This study shows that the two concentrations of *P. sidoides* extract tested are capable of delaying the *M. anisopliae* conidia germination speed, in comparison to the controls, although the germination frequency has been restored after about 12 h of conidia incubations, with did not lack conidia viability, indicating no toxicity.

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