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Toxicity study of the anti-hypertensive agent perindopril on the entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin assessed by conidia germination speed parameter

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Hypertension is an important vascular disease to the global public health, since it constitutes the principal cause of death from childhood to adulthood. In order to alleviate its symptoms, the treatment is accomplished by anti-hypertensive drugs, among them, is perindopril, an angiotensin-converting enzyme (ACE) inhibitor. The entomopathogenic filamentous fungus *Metarhizium anisopliae* is widely used for biological control and it has been promising in toxicity studies of substances assessed by conidia germination speed parameter. This study aimed to verify the effect of different concentrations of perindopril on the conidia germination speed of the model fungus *M. anisopliae*, for detecting a possible toxic effect of this medicament in another eukaryote. Conidia of *M. anisopliae* were incubated with perindopril in concentrations of 200 and 20 µg/ml at 28°C for 12 h, sampled at 0, 6, 8, 10 and 12 h and analyzed by light microscopy. The frequency of dormant, embedded, bud and germinated conidia was counted. As a result, perindopril in concentrations of 200 and 20 µg/ml increased the germination speed of *M. anisopliae* conidia, when compared to the negative control (absence of perindopril). It indicates that these two concentrations of perindopril have no toxicity on *M. anisopliae*, considering the Bayesian analysis.

Key words: Angiotensin-converting enzyme inhibitor, vegetative development, model fungus, entomopathogen, Bayesian analysis.

INTRODUCTION

The prevention of vascular diseases associated to hypertension became a priority question in global-health politics (MacMahon et al., 2008). In infancy, the occurrence of arterial hypertension varies between 1-3% and it increases to about 10% in adolescence, especially in obese patients (Dios, 2011). Cardiovascular diseases are the principal cause of death among adults in developed countries and there is an increase of them in developed countries and there is an increase of them in developing countries (Thom et al., 1998).

The estimative is that blood-pressure-related diseases kill around 8 million people per annum (Lawes et al., 2008). In Brazil, cardiovascular diseases are already the principal cause of death, being responsible for 30% of deaths in 2008; especially, the systemic arterial hypertension (SAH) affects more than 30 million Brazilians. In

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2010, the number of people affected by SAH in the United States reached 74 million (Giollo et al., 2010).

The antihypertensive pharmacotherapy uses different drug classes, classified as diuretics, adrenergic inhibitors, direct acting vasodilators, calcium channel blockers, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II (AT1 subtype) receptor antagonists and renin inhibitors (Giollo et al., 2010).

ACE inhibitors are widely employed as antihypertensive agents, not only for reducing systemic vascular resistance in hypertension patients but also to prevent and treat cardiovascular, renal and retinal diseases in patients with normal blood pressure (Wang et al., 2012), and it has been suggested that they decrease the risk of cancer (Lever et al., 1998; Yoshiji et al., 2001).

The anti-hypertensive agent perindopril is a long-acting and centrally active ACE inhibitor and according to the Perindopril Protection Against Recurrent Stroke Study (PROGRESS) Collaborative Group (2001) it has also been reported to reduce risks of dementia and cognitive decline in the patients with recurrent strokes. Moreover, a study conducted by Yamada et al. (2010), using a mouse model of Alzheimer's disease, indicates that oral administration of perindopril (0.1, 0.3 or 1 mg/kg/day) significantly ameliorates the cognitive impairment; the dosing regimen of 1 mg/kg/day inhibited the plasma ACE activities by more than 90% and the brain ACE activities by more than 50%.

The asexual filamentous fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin is capable of infecting more than 300 species of insect-pests. It was used for the first time as a microbial control agent of insects by Elie Metschnikoff, in 1879, to control the wheat grain beetle (*Anisoplia austriaca*) and afterward, it was used to control the sugar beet curculio (*Cleonus punctiventris*) (Roberts and St. Leger, 2004). Several products based on *Metarhizium* spp. strains have been developed in past years (Copping, 2004), both for application in biological control and in the production of drug substances such as antibiotics and immunomodulators (Isaka et al., 2005).

The asexual sporulation is a common mode of reproduction for several filamentous fungi (Osherov and May, 2000). For insect-pathogenic fungi such as *M. anisopliae*, asexual spores or conidia represent the infective unit and the inoculum source in the field after application in biological control (Alves et al., 2011). Conidia germination represents a crucial step to create enough penetration sites, ensuring the success of these fungi during the infection of their hosts. After a successful germination and penetration, the speed and intensity of the vegetative growth determine the virulence of entomopathogens (Schumacher and Poehling, 2012).

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). Moreover, optimum conditions such as temperature, humidity, pH and nutrient sources are essential for the conidia germination.

To use the germination speed as parameter, conidia are inoculated into a liquid medium and samples are collected periodically for counting and the number of germinated conidia is determined (Milner et al., 1991). Germination speed of *M. anisopliae* conidia has been used as parameter to evaluate the toxicity of substances, as employed by Alves et al. (2011) to assess the toxicity of the insect growth regulator lufenuron. Different parameters, including conidia germination, were used by Schumacher and Poehling (2012) to assess the effects of five pesticides on two strains of *M. anisopliae*.

According to the study of Rangel et al. (2004), the conidial germination parameter is employed to determine whether the substrate on which conidia were produced has influence on the endogenous reserves stored in conidia during conidiogenesis. Therefore, considering the conidia germination of *M. anisopliae* as a response due to the abiotic factors of environment, this study aimed to verify the effect of different concentrations of perindopril on the conidia germination speed of the model fungus *M. anisopliae*, for detecting a possible toxic effect of this medicament in a eukaryote.

MATERIALS AND METHODS

Fungal strain and culture media

Mato Grosso (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 perindopril

The MT strain was grown on Petri dishes containing CM (20 ml) at 28°C in biological oxygen demand (BOD). Conidia were obtained directly from seven to ten days-old sporulating cultures by scraping and suspended in aqueous solution of 0.01% Tween 80 (7 ml). This conidia solution was filtered through a glass funnel containing autoclaved gauze and it was added to saline solution (9 ml), obtaining a solution with a concentration of 1.01×10^7 conidia/ml. Into three glass flasks (10 ml) were inoculated 300 µl of conidia solution. Treatment 1 (T1) and treatment 2 (T2) received perindopril solution (perindopril diluted in LCM) in concentration of 200 and 20 µg/ml, respectively. The volume of both flasks was completed to 10 ml with LCM. In the control (C), only LCM (9.7 ml) was added.

Samples of C, T1 and T2 were collected in Eppendorf® microtubes and incubated in BOD at 28°C for 12 h. Samples were analyzed in triplicate at 0, 6, 8, 10 and 12 h of incubation. Germinated conidia were counted using Neubauer hemocytometer. A conidium was considered germinated when a germ-tube projected from it (Milner et al., 1991). The percentage germination

Table 1. Percentage of germinated *M. anisopliae* conidia in control and treatments throughout the incubation period.

Time/ phase	Dormant conidia			Embedded conidia			Bud conidia			Germinated conidia		
Flask	**C**	**T1**	**T2**	**C**	**T1**	**T2**	**C**	**T1**	**T2**	**C**	**T1**	**T2**
0 h	100	100	100	0	0	0	0	0	0	0	0	0
6 h	22.4	5.1	8.8	27.7	18.7	16.1	31.2	21.5	38.3	18.7	9.1	36.8
8 h	6.8	3.7	2.3	14	6.8	7.2	16.8	11	12.6	62.4	78.4	77.9
10 h	2.0	1.6	0.8	4.5	2.9	1.1	10	15	6.4	83.5	80.2	91.6
12 h	0	1.0	0	3	3.4	0.8	6.2	12.4	6.8	90.7	83.2	92.3

and germination speed were assessed by randomly observing 300 conidia.

Statistical analysis

As the Bayesian statistics is an approach that can handle small data sets and non-normal distributions (Alves et al., 2011), it was considered in order to study the behavior of conidia germination when the perindopril drug is present.

The conidia counting data was analyzed using the statistical package BRugs developed for R software (2008), and the Poisson distribution was assumed for the response variable. For each parameter, 10,000 values were generated in an MCMC (*Monte CarloMarkov Chain)* process, with a burn-in of 1000 initial values, and thinning interval of 10. The multiple comparisons procedure was based on the *a posteriori* samples of the estimates of the parameters. Significant differences were considered at the level of 5% between the treatments and control group 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.

A model of logistic regression was fitted to study the behavior of conidial germination over time. Data were analyzed using the same package and software described above. The binomial distribution was considered for the data of germination percentage, and the following formula was used:

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

For each parameter, 50,000 values were generated in an *Monte CarloMarkov Chain* (MCMC*)* process, considering a sample discard period of 5000 initial values, and thinning interval of 10. 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, generally the conidia germination percentage is given by:

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

With *θij* as the percentage of germinated conidia.

RESULTS AND DISCUSSION

Although fungi were at first time considered to belong to the kingdom of plants, Wainright et al. (1993) and Baldauf and Palmer (1993) pointed that comparative analyses of ribosomal RNA and protein sequences have proved that this organisms are even more closely related to animal cells than was previously known. In 1974, Smith and Rosazza suggested the definition of microbial systems as those that could mimic the biotransformations observed in mammals (Cerniglia, 1997). Also, considering the amenability of fungi to classical and molecular techniques, fungi are considered as model systems for studying fundamental cell biological questions, since basic principles of many cellular processes are conserved between fungi and animals (Steinberg and Perez-Martin, 2008). Therefore, the Mato Grosso strain of *M. anisopliae* was chosen as model system to evaluate the toxic effects of the anti-hypertensive agent perindopril.

The Bayesian analysis of MT conidia germination showed the existence of a statistically significant difference between the germination speed of control and treatments (Tables 1, 2, 3 and 4), mainly for T2 (20 µg/ml of perindopril). According to the study of Alves et al. (2011), this statistical method is an approach that can work on datasets considering the true distribution, being reliable for small groups of data.

At the end of incubation time, it was possible to observe that T2 induced an increase in the number of germinated conidia (92.3%) compared to C (90.7%) and T1 (83.2%) (Table 1). Also, according to the curve of germination speed (Figure 1), the conidia germination started approximately between 2 and 3 h of incubation for T2 and about 3-4 h for C and T1.

The means and ICr for counting of germinated conidia are shown in Table 2. A Bayesian ICr of 95% was considered, in which 95% of samples are contained, and smaller the interval, less dispersed is the parameter. The means of germinated conidia in 12 h were: 85.76 (C), 96.32 (T1) and 113.80 (T2), with a credibility interval formed by 2.5 and 97.5%.

The means of conidia germination in each incubation period sampled throughout the 12 h and the credibility intervals are detailed in Table 3. According to these results, the incubation period of 10 and 12 h were statistically equal, showing a higher germination percentage.

Table 2. Bayesian estimates for the counting of germinated *M. anisopliae* conidia in the presence of different concentrations of perindopril.

Treatment	Mean	Standard error	95% ICr	
			2.50%	97.50%
Control[c]	85.76	0.07292	80.93	90.33
200 µg/ml (T1)[b]	96.32	0.07639	91.17	101.30
20 µg/ml (T2)[a]	113.80	0.08820	108.80	119.30

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

Table 3. 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%
0	1.095e-03[d]	0.0002771	0.000	4.793e-03
6	6.701e+01[c]	0.0586000	61.700	7.253e+01
8	8.952e+01[b]	0.0706200	83.530	9.592e+01
10	1.725e+02[a]	0.0879500	163.700	1.814e+02
12	1.644e+02[a]	0.1044000	155.700	1.729e+02

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

Table 4. Bayesian estimates for the logistic regression coefficients for control and treatments.

Parameter	b0	b1	b2	r^2
C	-6.8590	1.4240	-0.04753	0.994821
T1	-1.74e+01	2.512e+00	-1.132e01	0.9987819
T2	-6.4410	1.5640	-0.05422	0.9970927

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

The periods of 0, 6 and 8 h were statistically different, showing low germination percentage.

The logistic regression adjusted effectively the percentage of germinated conidia over time, with average r^2 of 0.997. The values of regression coefficients for each treatment, with their respective r^2 can be seen in Table 4.

Similarly, the germination speed of *M. anisopliae* conidia was employed by Alves et al. (2011) as parameter to assess the toxicity of different concentrations of lufenuron, an insect development inhibitor/ insect growth regulator. Conidia of MT strain were incubated at 28°C and sampled through 12 h. The Bayesian analysis showed that lufenuron do not inhibited the MT conidia in concentrations of 700 /ml and 1 mg/ml, whereas the inhibition occurred with a concentration of 2 mg/ml. These authors concluded that the two low concentrations tested had no toxicity on *M. anisopliae*, suggesting the use of lufenuron in biological-chemical combinations with a low environmental impact to combat insect-pests, maintain viable the fungal inoculums after this application in field.

Among other parameters, the conidia germination speed of two *M. anisopliae* strains was applied recently, by Schumacher and Poehling (2012), to evaluate the effects of pesticides fipronil, permethrin, imidacloprid, amitraz and NeemAzal (in concentrations of 0.32, 1.6, 8.0, 40, and 200 ppm). Also, permethrin and imidacloprid were combined in a ratio of 5:1 and tested in four combinations (1.6 ppm permethrin and 0.32 ppm imidacloprid; 8 ppm permethrin and 1.6 ppm imidacloprid; 40 ppm permethrin and 8 ppm imidacloprid; 200 ppm permethrin and 40 ppm imidacloprid). Analysis of variance showed that the maximum inhibition of germination caused by these pesticides was ≤ 15% and most of the pesticides had no negative influence on the germination. It was concluded that the low of the five pesticides dosages dissolved in 1% DMSO (dimethyl sulfoxide) were compatible with *M. anisopliae* for an integrated pest management approach.

The conidia germination speed of *M. anisopliae* strains has been employed as parameters in studies about solar ultraviolet radiation. Rangel et al. (2004) examined the

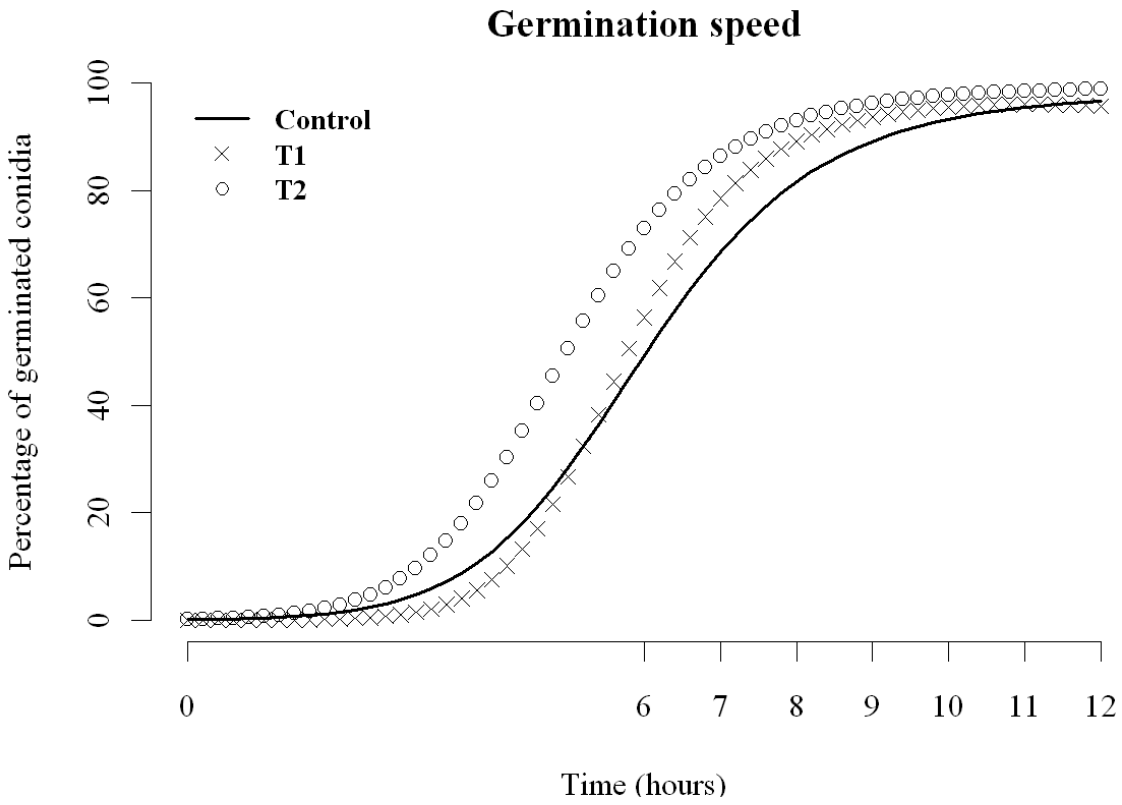

Figure 1. The curve of germination speed of *Metarhizium anisopliae* conidia in the control and treatments.

influence of growth substrate and nutritional environment on the conidial UV-B tolerance of *M. anisopliae* var. *anisopliae*, observing that conidia from insect cadavers germinated slower than those from PDAY culture medium. Rangel et al. (2005) observed that conidia produced on artificial or natural substrates have a similar culturability and tolerance to UV-B radiation, but conidia produced on Czapek's and Emerson's YpSs agar media or rice grains had higher tolerance to UV-B and germinated faster than conidia raised on PDA and PDAY media.

A wild and a transformant strain of *Penicillium roqueforti* were selected by García-Rico et al. (2009) to evaluate the effect of a heterotrimeric G protein α subunit on three parameters: conidia germination, stress res-ponse, and roquefortine C production. Conidia were incubated at 28°C for 14-16 h and sampled at regular intervals every 1-2 h, and then the numbers of conidia and of germinated conidia were counted. As results, at 12 h of incubation, the germination of the parental (wild) strain was very low (2.4% of the total number of conidia), whereas the conidia germination of the transformant strain was about 15% and these differences were main-tained throughout the observation period (6 vs. 36% at 36 h). As conclusion, these authors suggested that G protein-mediated signaling participates in the regulation of these three parameters assessed in *P. roqueforti*.

Pramanik et al. (2010) examined the effects of metabolism of antihistamine drug clemastine on the fungus model *Aspergillus niser* and compared the effect of metabolism with that of human volunteers, using HPLC analysis. As result, it was established that *A. niser* can be a potential model organism for drug metabolism study.

In the present study, the active compound perindopril accelerated the germination speed of conidia (at 6-8 h of incubation). Based on the obtained results, it is possible to conclude that perindopril has no toxicity considering the MT conidia germination speed as parameter. Considering the validity of filamentous fungi as model systems, these results are important data on the toxicity of perindopril and may be associated with other results already obtained for this drug, such as the review of the genotoxicity of marketed pharmaceuticals, published by Snyder and Green (2001), in which perindopril was pointed as negative for tests with following parameters: bacterial mutation, *in vitro* and *in vivo* cytogenetics, mouse lymphoma assay and carcinogenicity.

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