TOXICITY OF β-(1→3,1→6)-D-GLUCANS PRODUCED BY *Diaporthe* sp. ENDOPHYTES ON *Metarhizium anisopliae* (METSCHNIKOFF) SOROKIN ASSESSED BY CONIDIA GERMINATION SPEED PARAMETER

TOXICIDADE DE B-(1→ 3,1→ 6)-D-GLUCANAS PRODUZIDAS POR ENDÓFITOS Diaporthe sp. *SOBRE* Metarhizium anisopliae *(METSCHNIKOFF) SOROKIN AVALIADA PELO PARÂMETRO DE VELOCIDADE DE GERMINAÇÃO DOS CONÍDIOS*

Ravely Casarotti ORLANDELLI[1]; Tiago Tognolli de ALMEIDA[2]; Daniela Andressa Lino LOURENCO[3]; Ana Flora Dalberto VASCONCELOS[4]; Maria de Lourdes CORRADI DA SILVA[4]; João Lúcio de AZEVEDO[5]; João Alencar PAMPHILE[1*]

1. Universidade Estadual de Maringá (UEM), Maringá, PR, Brasil; 2. Universidade Católica Dom Bosco (UCDB), Campo Grande, MS, Brasil; 3. University of Georgia (UGA), Atenas, GA, Estados Unidos; 4. Faculdade de Ciências e Tecnologia, Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Presidente Prudente, SP, Brasil; 5. Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ/USP), Piracicaba, SP, Brasil; Núcleo Permanente Docente do Programa de Pós-graduação em Biotecnologia Ambiental da Universidade Estadual de Maringá (UEM), Maringá, PR, Brasil.
*prof.pamphile@gmail.com

ABSTRACT: We have previously reported that β-(1→3,1→6)-D-glucans produced by endophytes *Diaporthe* sp. G27-60 and G65-65 (GenBank accession codes JF766998 and JF767007, respectively) are promising anti-proliferation agents against human breast carcinoma (MCF-7) and hepatocellular carcinoma (HepG2-C3A) cells. However, the literature fails to describe the effects of *Diaporthe* exopolysaccharides (EPS) on eukaryotic healthy cells. The fungus *Metarhizium anisopliae* has been employed as model-system to evaluate the toxicity of pharmaceutical and agricultural-interest substances, taking into account, among other parameters, the speed of conidia germination. Current study verified the effect of different concentrations of *Diaporthe* β-glucans on the germination speed of *M. anisopliae*. Conidia were incubated with β-glucans treatments (50, 200 and 400 μg/mL) at 28ºC, sampled during 24 h and analyzed by light microscopy. At the end of a 24-h incubation, the amount of germinated conidia reached ≈99% for controls and ranged between 97.7 and 98.6% for treatments. Bayesian analysis indicated that *Diaporthe* glucans had no toxicity on *M. anisopliae* and the curve of germination occurred as expected for this fungal strain. Considering the validity of filamentous fungi as model-systems, results are important data on the toxicity of endophytic EPS on healthy cells and may be associated with our previous results obtained for these polymers against tumor cells.

KEYWORDS: Bayesian analysis. Endophytic fungi. Exopolysaccharide. Fungal model-system.

INTRODUCTION

The medicinal plant *Piper hispidum* Sw. (called *cordoncillo* in Mexico and *falso-jaborandi* in Brazil) harbors a diversity of endophytic fungi that inhabit the interior of the host plant without causing any damage (Orlandelli et al. 2012a), which include strains that secrete compounds with antimicrobial and enzymatic activity (Orlandelli et al. 2012b, 2015, 2017a). In a previous paper (Orlandelli et al. 2016), we identified that some strains are exopolysaccharide (EPS) producers: two of them, identified as *Diaporthe* (= *Phomopsis*) sp., secrete high-molecular weight EPS characterized as β-(1→3,1→6)-D-glucans, with potent anti-proliferation effects on tumor cells: inhibition ratios up to 74.6% and 83.3% against human breast carcinoma (MCF-7) and hepatocellular carcinoma (HepG2-C3A) cells, respectively (Orlandelli et al. 2017b).

Literature data report that *Phomopsis* (= *Diaporthe*) *foeniculi*, a phytopathogen isolated from fennel, secreted two EPS (a galactan and a mannan) that exhibited toxic effects (i.e. chlorosis, necrosis and/or wilting) on fennel, tobacco and tomato plants (Corsaro et al. 1998). Different biological systems could be evaluated to provide an in-depth investigation on the toxic effects of these polymers on healthy cells.

In 1974, Smith and Rosazza suggested that microbial systems could be defined as those capable of mimicking the biotransformations reported in mammals (Cerniglia 1997). Once the basic principles of many cellular processes are conserved between animals and fungi, these microorganisms may be used for studying fundamental cell

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biological issues, with the advantage of their amenability to classical and molecular techniques (Steinberg and Perez-Martin 2008). So that conidia germination may be employed as a parameter, fungal conidia are inoculated into a liquid medium; samples are periodically collected, and the number of germinated conidia is counted (Milner, Huppataz and Swaris 1991) to determine whether chemical variables influence fungal development and conidiogenesis (Rangel et al. 2004).

Metarhizium anisopliae cells may be used as model-system for toxicity assays of chemical products (Almeida et al. 2014). In this context, the germination speed of *M. anisopliae* conidia has been employed to evaluate the effects of nutritional and physical factors, and the toxicity or compatibility of pharmaceutical and agricultural-interest substances (Rangel, Alston and Roberts 2008, Alves et al. 2011, Akbar et al. 2012, Tonussi et al. 2012, Bulla et al. 2013, Fabrice et al. 2013, Almeida et al. 2014, Mochi et al. 2017, Sohrabi et al. 2019). Current study employs *M. anisopliae* conidia germination speed as a parameter for the evaluation of the toxicity of β-(1→3,1→6)-D-glucans secreted by *Diaporthe* sp. G27-60 and *Diaporthe* sp. G65-65, and expects to detect a possible toxic effect of the fungal polymers against eukaryotic healthy cells.

CONTENTS

The Mato Grosso (MT) strain of *M. anisopliae* var. *anisopliae* – retrieved from the Collection of Endophytic and Environmental Microorganisms, Laboratory of Microbial Biotechnology, Universidade Estadual de Maringá, Brazil (CMEA/LBIOMIC-UEM) – was used as a model-system to evaluate the possible toxic effects of different concentrations of β-glucans. The strain was grown on Petri dishes containing Complete medium (CM) (Pontecorvo et al. 1953), whilst conidia were obtained directly from seven-day-old sporulation cultures by scraping and suspended in a 0.01% Tween 80 aqueous solution (7 mL). The conidia solution was filtered through a glass funnel with autoclaved gauze and added to a saline solution (9 mL) to obtain a solution with a concentration of 3×10^7 conidia/mL. Further, 300 µL of conidia solution were inoculated into 10-mL glass flasks containing 400 µL of Liquid complete medium (LCM) (Pontecorvo et al. 1953). Each flask received 100 µL of β-glucan solution, as shown in Table 1.

Table 1. Treatments of β-(1→3,1→6)-D-glucans used in this study.

Treatment code	Concentration (µg/mL)	Glucan code	Glucan-producing endophyte
T1	50	EPS-P_{D1}	*Diaporthe* sp. G27-60
T2	200	EPS-P_{D1}	*Diaporthe* sp. G27-60
T3	400	EPS-P_{D1}	*Diaporthe* sp. G27-60
T4	50	EPS-P_{D2}	*Diaporthe* sp. G65-65
T5	200	EPS-P_{D2}	*Diaporthe* sp. G65-65
T6	400	EPS-P_{D2}	*Diaporthe* sp. G65-65

*Glucans were dissolved in DMSO (dimethyl sulfoxide) and diluted with Liquid complete medium (final DMSO concentration ≤1%).

The β-(1→3,1→6)-D-glucans were previously obtained by Orlandelli et al. (2017b) from submerged cultures of *Diaporthe* sp. G27-60 and *Diaporthe* sp. G65-65 (GenBank codes JF766998 and JF767007, respectively), two endophytes isolated from the medicinal plant *Piper hispidum* Sw. (Orlandelli et al. 2012) and retrieved from the CMEA/LBIOMIC-UEM. Controls comprised (C1), namely, β-glucan solution replaced by same volume of water, or (C2) LCM plus dimethyl sulfoxide (DMSO; Sigma-Aldrich Co.) at concentration 1%. The three concentrations (50, 200 and 400 µg/mL) of β-glucans tested were prepared under same conditions previously described by (Orlandelli et al. 2017b) for anti-proliferation assay and, then, dissolved in DMSO and diluted with culture medium (LCM). The DMSO concentration used (≤1%) has already been reported as compatible with *M. anisopliae* conidia germination (Schumacher and Poehling 2012, Wenzel Rodrigues et al. 2017).

All flasks remained incubated at 28±2ºC for 24 h and triplicates of control and treatment samples were collected periodically (at 0, 6, 8, 10, 12 and 24 h). Conidia were counted using a Neubauer hemocytometer and light microscopy, and the percentage of germinated conidia was assessed by randomly observing 100 conidia per sample. A conidium was considered germinated when a germ-tube was projected (Milner et al. 1991).

So that possible differences could be verified in the conidia germination among treatments and control groups, incubation time and their interactions, the counting data were analyzed with statistical package BRugs for software R (R Development Core Team 2008) and the Poisson

distribution was assumed, implemented by Bayesian methodology. The Monte CarloMarkov Chain (MCMC) was composed of 10,000 samples for each parameter, with a burn-in period of 1,000 initial values and thinning interval of 10, or rather, at every 10 values generated, one belonged to the sample, with 900 values generated. Significant differences were considered at 5% level between treatments if the zero value was not included in the credibility interval of the desired contrast. A non-informative Gamma distribution was considered *a priori* for germinated conidia averages, that is, $\theta_n \sim G(10^{-3};10^{-3})$, where θ_n is the mean for each n treatment considered.

The analysis of the behavior of conidia germination over time consisted of a model of logistic regression, whilst data were analyzed with the same package and software described above. The binomial distribution was considered for the data of germination percentage, and the following formula (1) was used:

$$\log it(\theta ij) = \beta_0 + \beta_1 time + \beta_2 time^2 \quad (1)$$

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 time.

The regression fit was tested by the coefficient of determination (r^2) and binomial distribution was taken into account for germination percentage data. Further, 10,000 values were generated in a MCMC process for each parameter, considering a sample discard period of 1,000 initial values. The final sample was taken with steps of 10. Logistic regression coefficients were significant at 5% level 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, or rather, 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 the formula (2) for quadratic regressions:

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

where θij is the percentage of germinated conidia.

Herein, the MT conidia germination speed represented a rapid and alternative toxicity assay which significantly expanded investigation on toxicity studies. Comparative analyses of ribosomal RNA and protein sequences proved that fungi are closely related to animal cells (Baldauf and Palmer 1993, Wainright et al. 1993). Furthermore, fungi may be easily grown and manipulated under laboratory conditions for studying cellular and genetics processes (Kibbler et al. 2018), justifying the choice of current fungal model-system.

Approximately 16% of conidia were germinated at 6 h (Table 2), and significantly increased at 8 h (~66%), corroborating the curve of germination speed (Figure 1), which shows that, for all controls and treatments, the conidia germination started close to 4 - 6h of incubation. It was more apparent at 8 h, as expected for this fungal strain in the absence of toxic substances (Alves et al. 2011). At the end of 24 h of incubation, the amount of germinated conidia was ≥99.0% for controls, and ranged between 97.7% and 98.6% for treatments. Germination speed curve indicated that no delays on fungal germination occurred when MT conidia was treated with *Diaporthe* β-glucans at 50, 200 and 400 µg/mL concentrations.

Table 2. Percentage (mean of triplicates ± standard deviation) of germinated *Metarhizium anisopliae* conidia in control and β-glucans treatments throughout the incubation time.

Treatment	Incubation time (h)					
	0	**6**	**8**	**10**	**12**	**24**
C1	0.0±0.0	18±2.0	68.0±2.0	78.3±1.5	95.0±3.0	99.7±0.6
C2	0.0±0.0	16.0±2.0	67.0±1.0	76.0±1.0	93.0±1.0	99.0±1.0
T1	0.0±0.0	15.7±2.9	66.7±2.0	75.7±0.6	93.0±1.0	98.0±1.7
T2	0.0±0.0	15.0±2.6	65.3±0.6	75.3±2.1	92.3±2.5	98.3±0.6
T3	0.0±0.0	15.0±1.0	65.0±2.7	74.7±0.6	92.0±2.0	98.3±1.5
T4	0.0±0.0	16.0±1.0	65.7±2.1	75.3±0.6	92.7±1.5	98.6±0.6
T5	0.0±0.0	15.7±1.1	65.7±1.1	75.0±1.0	92.3±2.1	98.0±1.0
T6	0.0±0.0	15.3±0.6	65.3±2.3	74.3±0.6	92.0±1.0	97.7±2.5

C1 = water, C2 = Liquid complete medium plus DMSO at the concentration of 1%. β-glucan produced by *Diaporthe* sp. G27-60: T1 = 50 µg/mL, T2 = 200 µg/mL, T3 = 400 µg/mL. β-glucan produced by *Diaporthe* sp. G65-65: T4 = 50 µg/mL, T5 = 200 µg/mL, T6 = 400 µg/mL.

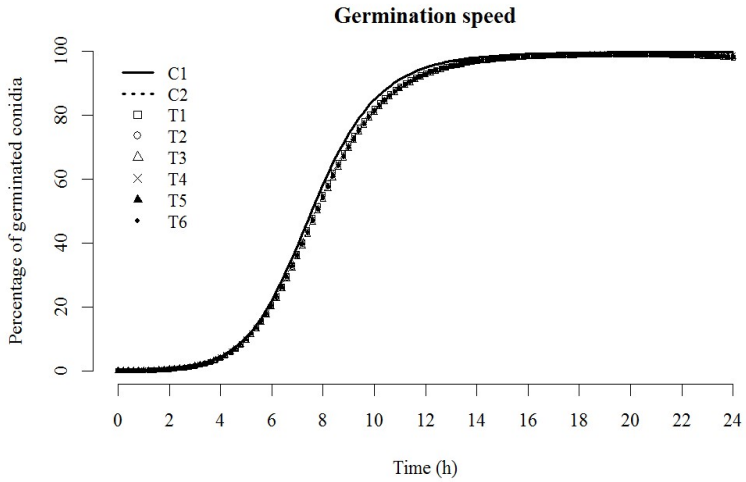

Figure 1. The curve of germination speed of MT strain of *M. anisopliae* conidia in the controls and β-glucan treatments. C1 = water, C2 = Liquid complete medium plus DMSO at the concentration of 1%. β-glucan produced by *Diaporthe* sp. G27-60: T1 = 50 µg/mL, T2 = 200 µg/mL, T3 = 400 µg/mL. β-glucan produced by *Diaporthe* sp. G65-65: T4 = 50 µg/mL, T5 = 200 µg/mL, T6 = 400 µg/mL.

The spore germination is a process that comprises a sequence of events that activate the resting spore (D'Enfert 1997). When triggered to germinate, the cell becomes hydrated and there is a marked increase in respiratory activity, followed by a progressive increase in the synthesis of protein and nucleic acids (Deacon 2006). The resting spore is subsequently converted into a rapidly growing germ-tube from which the mycelium is formed by elongation and branching (D'Enfert 1997, Deacon 2006). 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 conidia germination (Almeida et al. 2014). The process is directly affected by nutritional, environmental, physical and chemical factors (Rangel et al. 2004). Variations in the speed of conidia germination may be observed in response to different stress conditions (Roberts and St Leger 2004, Rangel, Alston and Roberts 2008).

The Bayesian analysis is an approach that works on datasets with true distribution, being reliable for small groups of data (Alves et al. 2011). It has been used to obtain precise estimates without needing any kind of transformation (Gomes et al. 2014). The statistical method revealed the absence of a statistically significant difference between the germination speed of controls and treatments. A Bayesian ICr of 95% is the interval in which 95% of the samples are contained, and the smaller the interval, the less dispersed is the parameter. Overall (Table 3), the means of germinated conidia in the interval 0-24 h ranged between 57.40 and 59.75%. According to these results, the β-glucans had no inhibitory effect on the *M. anisopliae* germination when compared to controls.

Table 3. Bayesian estimates for the counting of germinated *M. anisopliae* conidia in controls and β-glucans treatments.

Treatment	Mean (%)	Standard error	95% ICr	
			2.50%	97.50%
C1	59.75[a]	0.06	56.53	63.37
C2	58.63[a]	0.06	55.02	62.05
T1	58.04[a]	0.06	54.56	61.79
T2	57.67[a]	0.06	54.27	61.19
T3	57.40[a]	0.06	53.70	61.02
T4	58.06[a]	0.06	54.58	61.85
T5	57.76[a]	0.07	54.37	61.23
T6	57.42[a]	0.06	57.36	61.12

[a]Same letter indicates that means of germinated conidia do not differ according to the Bayesian analysis. C1 = water, C2 = Liquid complete medium plus DMSO at the concentration of 1%. β-glucan produced by *Diaporthe* sp. G27-60: T1 = 50 µg/mL, T2 = 200 µg/mL, T3 = 400 µg/mL. β-glucan produced by *Diaporthe* sp. G65-65: T4 = 50 µg/mL, T5 = 200 µg/mL, T6 = 400 µg/mL.

The means of germinated conidia significantly increased through the 0 - 12 h interval, while the incubation time of 12 and 24 h was statistically equal, showing higher germination percentages that reached a mean of 97.93% of conidia germinated at 24 h (Table 4). The germination speed remained increasing between 8 and 14 h of incubation, after which it became stable. The logistic regression adjusted efficiently the conidia germination percentage over time (Table 5), showing that the germination percentage over time showed a quadratic behavior.

Table 4. Means and credibility intervals for counting of the germinated *M. anisopliae* conidia throughout the incubation time.

Time (h)	Mean (%)	Standard error	95% ICr	
			2.50%	97.50%
0	00.01[e]	3.72e-4	2.46e-19	0.04
6	15.71[d]	0.03	14.26	17.33
8	65.72[c]	0.06	62.30	69.06
10	75.38[b]	0.05	72.09	78.63
12	92.37[a]	0.07	88.57	95.98
24	97.93[a]	0.07	93.77	101.90

[a-e]Different letters indicate that the means of germinated conidia differ according to the Bayesian analysis.

Table 5. Bayesian estimates for the logistic regression coefficients for controls and β-glucans treatments.

Treatment	b_0	b_1	b_2	r^2
C1	-7.2940	1.1610	-0.0260	0.9805
C2	-7.3110	1.1650	-0.0280	0.9758
T1	-7.5420	1.2160	-0.0309	0.9752
T2	-7.4730	1.1920	-0.0297	0.9760
T3	-7.3670	1.1700	-0.0290	0.9754
T4	-7.2840	1.1600	-0.0282	0.9775
T5	-7.3920	1.1850	-0.0298	0.9764
T6	-7.3900	1.1830	-0.0300	0.9748

b_0 = intercept, b_1 = linear coefficient, b_2 = quadratic coefficient, r^2 = coefficient of regression determination. C1 = water, C2 = Liquid complete medium plus DMSO at the concentration of 1%. β-glucan produced by *Diaporthe* sp. G27-60: T1 = 50 μg/mL, T2 = 200 μg/mL, T3 = 400 μg/mL. β-glucan produced by *Diaporthe* sp. G65-65: T4 = 50 μg/mL, T5 = 200 μg/mL, T6 = 400 μg/mL.

Conidial germination may be employed to determine whether the substrate on which conidia were produced affected the endogenous reserves stored in conidia during conidiogenesis (Rangel et al. 2004). Corroborating what was reported above, Bulla et al. (2013) evaluated the toxicity of the anti-hypertensive agent perindopril on MT strain by conidia germination speed parameter; no toxicity was detected and concentrations of 200 and 20 μg/ml increased the germination speed. On the contrary, Almeida et al. (2014) reported that EPs 7630® (an ethanolic root extract from the plant *Pelargonium sidoides*) delayed MT conidia germination when compared to controls, although the conidia viability was preserved.

CONCLUSIONS

Current study is the first report on possible toxic effects of β-glucans from *Diaporthe* strains against eukaryotic healthy cells. Based on the obtained results, it may be concluded that β-(1→3,1→6)-D-glucans have no toxicity when conidia germination speed is taken as parameter. Considering the validity of filamentous fungi as model systems, these results are important data on the toxicity of fungal EPS on healthy cells and may be associated with other results already obtained for these polymers against tumor cells.

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RESUMO: Anteriormente, um estudo mostrou que β-(1→3,1→6)-D-glucanas produzidas pelos endófitos *Diaporthe* sp. G27-60 e G65-65 (códigos de acesso no GenBank JF766998 e JF767007, respectivamente) são agentes promissores com ação antiproliferativa contra células HepG2-C3A (hepatoma humano) e MCF-7 (adenocarcinoma mamário humano). No entanto, os efeitos de exopolissacarídeos (EPS) produzidos por fungos do gênero *Diaporthe* em células eucarióticas sadias não estão descritos na literatura atual. O fungo *Metarhizium anisopliae* tem sido utilizado como sistema-modelo para avaliar a toxicidade de substâncias de interesse farmacêutico e agronômico, considerando, entre outros parâmetros, a velocidade de germinação de conídios. O presente estudo teve como objetivo verificar os efeitos de diferentes concentrações de β-glucanas produzidas por *Diaporthe* sp. sobre a velocidade de germinação de *M. anisopliae*. Os conídios foram incubados com os tratamentos de β-glucanas (50, 200 e 400 μg/mL) a 28 ºC, com amostras coletadas ao longo de 24 h, e analisados por microscopia de luz. Ao final das 24 h de incubação, o total de conídios germinados nos controles foi de ≈99%, e variou entre 97,7 e 98,6% para os tratamentos. A análise bayesiana indicou que as glucanas de *Diaporthe* sp. não apresentaram toxicidade sobre *M. anisopliae*, e a curva de germinação atendeu ao esperado para essa linhagem fúngica. Considerando a validade dos fungos filamentosos como sistemas-modelo, esses resultados representam dados importantes sobre a toxicidade dos EPS de endófitos sobre células sadias e podem ser associados aos resultados anteriormente obtidos para esses polímeros em testes contra células tumorais.

PALAVRAS-CHAVE: Análise bayesiana. Fungos endofíticos. Exopolissacarídeo. Sistema-modelo fúngico.

REFERENCES

AKBAR, S., et al. Compatibility of *Metarhizium anisopliae* with different insecticides and fungicides. *African Journal of Microbiology Research* [online]. May 2012, 6 (17), 3956-3962 [cited 2019-02-27]. Available from Internet: https://doi.org/10.5897/AJMR12.417. ISSN: 1996-0808.

ALMEIDA, T. T., et al. Sensibility of the entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin to *Pelargonium sidoides* extract (EPs 7630®) assessed by conidia germination speed parameter. *African Journal of Biotechnology* [online]. February 2014, 13 (7), 821-826 [cited 2019-02-15]. Available from Internet: https://doi.org/10.5897/AJB2013.13235. ISSN 1684-5315.

ALVES, M. M. T. A., et al. Toxicity of the insect growth regulator lufenuron on the entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin assessed by conidia germination speed parameter. *African Journal of Biotechnology* [online]. August 2011, 10 (47), 9661-9667 [cited 2019-02-10]. Available from Internet: https://www.ajol.info/index.php/ajb/article/view/95734I. ISSN 1684-5315.

BALDAUF, S. L. and PALMER, J. D. Animals and fungi are each other's closest relatives: congruent evidence from multiple proteins. *Proceedings of the National Academy of Sciences of the United States of America* [online]. December 1993, 90 (24), 11558-11562 [cited 2019-02-10]. Available from Internet: https://doi.org/10.1073/pnas.90.24.11558. ISSN 1091-6490.

BULLA, L. M. C., et al. Toxicity study of the anti-hypertensive agent perindopril on the entomopathogenic fungus Metarhizium anisopliae (Metschnikoff) Sorokin assessed by conidia germination speed parameter. *African Journal of Biotechnology* [online]. August 2013, 12 (35), 5452-5457 [cited 2019-02-15]. Available from Internet: https://doi.org/10.5897/AJB12.1810. ISSN 1684-5315.

CERNIGLIA, C. E. Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation. *Journal of Industrial Microbiology and Biotechnology* [online]. November 1997, 19 (5-6), 324-333 [cited 2019-02-23]. Available from Internet: https://doi.org/10.1038/sj.jim.2900459. ISSN 1476-5535.

CORSARO, M. M., et al. Chemical structure of two phytotoxic exopolysaccharides produced by *Phomopsis foeniculi*. *Carbohydrate Research* [online]. April 1998, 308 (3-4), 349-335 [cited 2019-02-23]. Available from Internet: https://doi.org/10.1016/S0008-6215(98)00085-8. ISSN 1873-426X.

D'ENFERT, C. Fungal spore germination: insights from the molecular genetics of *Aspergillus nidulans* and *Neurospora crassa*. *Fungal Genetics and Biology* [online]. April 1997, 21 (2), 163-172 [cited 2019-02-15]. Available from Internet: https://doi.org/10.1006/fgbi.1997.0975. ISSN 1096-0937.

DEACON, J. W. *Fungal biology*. 4th ed. Oxford: Blackwell Publishing, 2006. ISBN 978-1-4051-3066-0.

FABRICE, C. E. S., et al. Compatibility of entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin with fungicide thiophanate-methyl assessed by germination speed parameter. *Journal of Food, Agriculture and Environment* [online]. January 2013, 11 (1), 368-372 [cited 2019-02-27]. Available from Internet: https://doi.org/10.1234/4.2013.3885. ISSN 1459-0263.

GOMES, L. C., et al. The impact of dietary dry yeast on lactation curves of primiparous and multiparous Saanen goats. *Acta Scientiarum. Animal Science* [online]. October 2014, 36 (4), 405-411 [cited 2019-02-15]. Available from Internet: https://doi.org/10.4025/actascianimsci.v36i4.24446. ISSN 1806-2636.

KIBBLER, C. C., et al. (Ed). *Oxford textbook of medical mycology*. Oxford: Oxford University Press, 2018. ISBN 978-0198755388.

MILNER, R. J. and HUPPATAZ, R. J. and SWARIS, S. C. A new method for assessment of germination of *Metarhizium* conidia. *Journal of Invertebrate Pathology* [online]. [cited 2019-02-10]. Available from Internet: https://doi.org/10.1016/0022-2011(91)90048-U. ISSN 1096-0805.

MOCHI, D. A., et al. Compatibility of *Metarhizium anisopliae* with liposoluble photoprotectants and protective effect evaluation against solar radiation. *Bioscience Journal* [online]. July 2017, 33 (4), 1028-1037 [cited 2019-02-27]. Available from Internet: https://doi.org/10.14393/BJ-v33n4a2017-34080. ISSN 1981-3163.

ORLANDELLI, R. C., et al. Diversity of endophytic fungal community associated with *Piper hispidum* Sw. (Piperaceae) leaves. *Genetics and Molecular Research* [online]. May 2012a, 11 (2), 1575-1585 [cited 2019-01-20]. Available from Internet: http://dx.doi.org/10.4238/2012.May.22.7. ISSN 1676-5680.

ORLANDELLI, R. C., et al. *In vitro* antibacterial activity of crude extracts produced by endophytic fungi isolated from *Piper hispidum* Sw. *Journal of Applied Pharmaceutical Science* [online]. October 2012b, 2 (10), 137-141 [cited 2019-01-20]. Available from Internet: https://doi.org/10.7324/JAPS.2012.21027. ISSN 2231-3354.

ORLANDELLI, R. C., et al. Antifungal and proteolytic activities of endophytic fungi isolated from *Piper hispidum* Sw. *Brazilian Journal of Microbiology* [online]. April 2015, 46 (2), 359-366 [cited 2019-02-10]. Available from Internet: https://doi.org/10.1590/S1517-838246220131042. ISSN 1678-4405.

ORLANDELLI, R. C., et al. Screening of endophytic sources of exopolysaccharides: preliminary characterization of crude exopolysaccharide produced by submerged culture of *Diaporthe* sp. JF766998 under different cultivation time. *Biochimie Open* [online]. June 2016, 2, 33-40 [cited 2019-02-23]. Available from Internet: https://doi.org/10.1016/j.biopen.2016.02.003. ISSN 2214-0085.

ORLANDELLI, R. C., et al. Use of agro-industrial wastes as substrates for α-amylase production by endophytic fungi isolated from *Piper hispidum* Sw. *Acta Scientiarum. Technology* [online]. July 2017a, 39 (3), 255-261 [cited 2019-02-23]. Available from Internet: https://doi.org/10.4025/actascitechnol.v39i3.30067. ISSN 1807-8664.

ORLANDELLI, R. C, et al. β-(1→3,1→6)-D-glucans produced by *Diaporthe* sp. endophytes: purification, chemical characterization and antiproliferative activity against MCF-7 and HepG2-C3A cells. *International*

Journal of Biological Macromolecules [online]. January 2017b, 94 (Pt A), 431-437 [cited 2019-02-10]. Available from Internet: https://doi.org/10.1016/j.ijbiomac.2016.10.048. ISSN 1879-0003.

PONTECORVO, G., et al. The genetics of *Aspergillus nidulans*. *Advances in Genetics* [online]. 1953, 5, 141-238 [cited 2019-02-10]. Available from Internet: https://doi.org/10.1016/S0065-2660(08)60408-3. ISSN 2159-1563.

R DEVELOPMENT CORE TEAM. *R: A language and environment for statistical computing*. Viena: R Foundation for Statistical Computing, 2008. ISBN 3-900051-07-0.

RANGEL, D. E. N., et al. Variations in UV-B tolerance and germination speed of *Metarhizium anisopliae* conidia produced on insect and artificial substrates. *Journal of Invertebrate Pathology* [online]. October 2004, 87 (2-3), 77-83 [cited 2019-01-20]. Available from Internet: https://doi.org/10.1016/j.jip.2004.06.007. ISSN 1096-0805.

RANGEL, D. E. N. and ALSTON, D. G. and ROBERTS, D. W. Effects of physical and nutritional stress conditions during mycelial growth on conidial germination speed, adhesion to host cuticle, and virulence of *Metarhizium anisopliae*, an entomopathogenic fungus. *Mycological Research* [online]. November 2008, 112 (11), 1355-1361 [cited 2019-02-27]. Available from Internet: https://doi.org/10.1016/j.mycres.2008.04.011. ISSN 1469-8102.

ROBERTS, D. W. and ST LEGER, R. J. *Metarhizium* spp., cosmopolitan insect pathogenic fungi: mycological aspects. *Advances in Applied Microbiology* [online]. 2004, v. 54, 1-70 [cited 2019-02-27]. Available from Internet: https://doi.org/10.1016/S0065-2164(04)54001-7. ISSN 0065-2164.

SCHUMACHER, V. and POEHLING, H. M. *In vitro* effect of pesticides on the germination, vegetative growth, and conidial production of two strains of *Metarhizium anisopliae*. *Fungal Biology* [online]. January 2012, 116, 121-132 [cited 2019-02-10]. Available from Internet: https://doi.org/10.1016/j.funbio.2011.10.007. ISSN 1878-6162.

SOHRABI, F., et al. Evaluation of the compatibility of entomopathogenic fungi and two botanical insecticides tondexir and palizin for controlling *Galleria mellonella* L. (Lepidoptera: Pyralidae). *Crop Protection* [online]. March 2019, 117, 20-25 [cited 2019-02-27]. Available from Internet: https://doi.org/10.1016/j.cropro.2018.11.012. ISSN 1873-6904.

STEINBERG, G. and PEREZ-MARTIN, J. *Ustilago maydis*, a new fungal model system for cell biology. *Trends in Cell Biology* [online]. 2008, 18 (2), 61-67 [cited 2019-02-23]. Available from Internet: https://doi.org/10.1016/j.tcb.2007.11.008. ISSN 1879-3088.

TONUSSI, R. L., et al. Toxicity of the pyrethroid deltamethrin on the entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae* (Metsch) Sorokin assessed by germination speed parameter. *Annals of Biological Research* [online]. 2012, 3 (11), 5028-5033 [cited 2019-02-27]. Available from Internet: https://www.scholarsresearchlibrary.com/abstract/toxicity-of-the-pyrethroid-deltamethrin-on-the-entomopathogenic-fungus-metarhiziumrnanisopliae-var-anisopliae-metsch-sor-7668.html. ISSN 0976-1233.

WAINRIGHT, P. O., et al. Monophyletic origins of the Metazoa: an evolutionary link with fungi. *Science* [online]. 1993, 260 (5106), 340-342 [cited 2019-02-10]. Available from Internet: https://www.jstor.org/stable/2881059. ISSN 1095-9203.

WENZEL RODRIGUES, I. M. et al. Compatibility of polymers to fungi *Beauveria bassiana* and *Metarhizium anisopliae* and their formulated products stability. *Acta Scientiarum. Agronomy* [online]. October 2017, 39 (4), 457-464 [cited 2019-02-10]. Available from Internet: https://doi.org/10.4025/actasciagron.v39i4.32903. ISSN 1807-8621.