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ARTICLE

Action of *Ganoderma lucidum* mycelial growth filtrates on *Erysiphe diffusa* and embryotoxicity assessment in a chicken embryo model

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ABSTRACT   This work aimed to evaluate the antimicrobial effect of *Ganoderma lucidum* mycelial growth filtrates (MGF) on the phytopathogen *Erysiphe diffusa* and their potential effects on the embryonic development of *Gallus gallus*. The antimicrobial activity was evaluated on *E. diffusa* spores by the microdilution broth method. To evaluate embryotoxic and teratogenic effects, fertile eggs of *G. gallus* received injections of solutions containing the filtrates of *G. lucidum* through the air chamber. After three days of incubation, we opened the eggs and evaluated egg viability, embryo survival, malformation occurrence, embryonic staging and heart rate. Live embryos were prepared using whole mount technique and the morphological analysis was performed. We used the generalized linear model to fit embryotoxicity and teratogenicity data. We verified that *G. lucidum* MGF showed inhibitory activity in vitro against *E. diffusa* and the minimum inhibitory concentrations ranged from 5 to 10 mg/mL. We could also observe that the filtrates did not present embryotoxic or teratogenic effects on the early embryonic development of *G. gallus,* but induced significant differences in the embryonic mean heart rate and on the stage of embryonic development.
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Introduction

Ganoderma lucidum is one of the best-known medicinal mushrooms and has been used by traditional medicine in Asian countries for thousands of years (Lu et al. 2020), mainly because of its therapeutic properties (Ahmad 2020). It has been reported that *G. lucidum* contains over 400 bioactive compounds, produced by spores, mycelia and fruiting bodies (Batra et al. 2013), like triterpenoids, polysaccharides, amino acids, enzymes and dietary fibers (Lakhanpal and Rana 2005; Yang et al. 2019). Although its therapeutic properties are widely studied, there are few works exploring its action over pathogenic species of agricultural interest. However, some studies have shown antimicrobial activity of *G. lucidum* against phytopathogens like *Pseudomonas syringae, Erwinia amylovora* and *Mycosphaerella fijiensis* (Ofodile et al. 2005; Perez-Holguin et al. 2017; Arias-Londoño et al. 2019).

Soybean powdery mildew, caused by the parasitic fungus *Erysiphe diffusa*, occurs in countries such as Brazil, United States, Paraguay and Bolivia (Dunn and Gaynor 2020). Initial symptoms are evidenced by the growth of a thin whitish layer on the leaves surface that can reduce productivity and lead to economic losses (Igarashi et al. 2010). Transmission of the disease can occur through the dispersion of spores and by infected seeds and plant remains, for example, which makes it difficult to control (Pérez-Vega et al. 2013). Application of fungicides is the most used control method (Perina et al. 2013); though, it can cause problems, such as the selection of resistant pathogenic strains and environmental contamination (Resende et al. 2009). Hence, the identification of antimicrobial compounds with unique and versatile aspects, such as low toxicity and antimicrobial potency is crucial (Jogaiah et al. 2019).

Despite the benefits of using alternatives to commercial synthetic phytosanitary products, certain substances present in so-called biopesticides, considered natural, can be harmful to non-target organisms, invertebrates or vertebrates (Yim et al. 2014; Machado et al. 2017). Because of that, several regulatory agencies recommend safety tests to assess possible harmful effects of these products on model organisms (Environmental Protection Agency 1996; Ministério da Agricultura, Pecuária e Abastecimento 2012; Organisation for Economic Co-operation and De-

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velopment 2012). Among the species most used as a model for vertebrates is the domestic chicken (*Gallus gallus*), since its manipulation in laboratory is quite common in tests for assessing the toxicity of biological and alternative pest control agents (Lim et al. 2012; Haas et al. 2017).

G. gallus embryo is considered one of the first model organisms used in the study of vertebrate embryonic development (Rallis 2007). Studies with similar methods to those used in this work have been carried out since the end of the 19th century, and *in ovo* testing of chemicals has expanded since the 1960s (McLaughlin et al. 1963). Many factors contribute to its use in research; among them are the molecular and cellular similarities with human embryos (Vergara and Canto-Soler 2012), its rapid and well-documented development, and low costs of acquisition (Schoenwolf 1999; Smith et al. 2012).

This study aimed to evaluate whether the mycelial growth filtrate of *G. lucidum* has an inhibitory effect *in vitro* on *E. diffusa* and the possible effects on the non-target organism *G. gallus* development.

Materials and Methods

Organisms

Federal University of Technology – Paraná Institutional Animal Care and Use Committee approved all the experimental procedures in this study (protocol n. 2018-10).

Ganoderma lucidum (Curtis) P. Karst., isolate CC339ST, was obtained from Brazilian Agricultural Research Corporation (Embrapa) – Genetic Resources and Biotechnology (Brasília, DF, Brazil). Inoculated Petri dishes with potato-dextrose-agar (PDA; Kasvi, Brazil) were incubated at 28 °C for seven days and stored at 4 °C. Mycelium was activated in PDA for 10 days following the same procedure and then used for inoculation in liquid medium. The isolate of *Erysiphe diffusa* (Cooke & Peck) U. Braun & S.Takam. was maintained in young soybean plants, *Glycine max* (L.) Merr, cultivar NA 5909.

For the embryotoxicity and teratogenicity tests, fertile eggs of layer chickens (*Gallus gallus* subsp. *domesticus* L., 1758), aged from 36 to 40 weeks, were purchased from a commercial hatchery located in Dois Vizinhos, PR, Brazil.

Submerged fermentation of Ganoderma lucidum

This process was performed according to Cruz et al. (2019). To obtain the filtrates, ten mycelial agar discs (5 mm in diameter) were transferred to Erlenmeyer flasks containing 200 mL of potato-dextrose (PD; Kasvi, Brazil) liquid culture medium, pH 5.1. For 15 days, the flasks were shaken on a rotary shaker, at 120 rpm, in the dark, at 28 ± 2 °C. After this period, the elicitors 2 mM salicylic acid (AS) and 5% lignin were dissolved in water, steril-

ized and subsequently added to the flasks containing *G. lucidum*. Elicitors are biotic or abiotic compounds capable of stimulating an organism's defense and they are used in liquid medium to increase the intensity of cellular responses and subsequent influence on the production of secondary metabolites (Murthy et al. 2014).

We performed three repetitions for each treatment, using nine Erlenmeyer flasks in total. The flasks containing the treatments with the elicitors were kept in an orbital shaker at 120 rpm, in the dark, at 28 ± 2 °C, for 50 days. In order to obtain *G. lucidum* mycelial growth filtrates (MGF), the media then were filtered through Whatman filter paper n. 41, to separate the mycelium. As a result, we obtained MGF of *G. lucidum* (i) without elicitation; (ii) elicited with lignin; and (iii) elicited with AS.

Determination of minimum inhibitory concentration (MIC)

In vitro antimicrobial activity on *E. diffusa* was evaluated using the microdilution broth method, following the M38-A standard, with some modifications, standardized by the Clinical and Laboratory Standards Institute (2002).

Following the Folin-Ciocalteu method described by Singleton and Rossi (1965), we determined the total phenolic content of *G. lucidum* samples. Gallic acid was used to obtain the standard curve (0.0094-0.15 mg/mL), and the results were expressed as mg of gallic acid equivalents (GAE) per mL of filtrate. In 96-well microdilution plates, 100 µL of PD culture medium and 200 µL of *G. lucidum* MGF were added and the initial concentration was based on values of phenolic compounds found in the filtrates, starting with 80 mg of GAE/mL. Each solution was pipetted only in the first well of the column and therefrom a serial dilution was performed, with the final concentrations obtained: 40, 20, 10, 5, 2.5, 1.25, 0.625, and 0.312 mg/mL. Then, 10 µL of a microbial suspension of *E. diffusa* prepared in saline solution with turbidity equivalent to 0.5 on the McFarland scale (1.0×10^6 CFU/mL) was added to each well. Distilled water was used as negative control and a commercial copper oxychloride-based fungicide (50 ppm) as positive control.

The microplates were kept in a growth chamber at 25 °C ± 1 °C, for 24 h. In order to reveal the results, 3 h before the end of the incubation, 10 µL of 0.01% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, USA) was added to each well. In the presence of microorganisms (live cells) a reduction reaction occurs, resulting in the formation of a red, stable and non-diffusible compound, known as triphenylformazan (Summanen et al. 1992). The minimum inhibitory concentration was considered as being the lowest concentration capable of inhibiting the visible growth of the fungus.

Analysis of the effects on the early embryonic development of G. gallus

After a disinfecting process with 70% ethyl alcohol and ultraviolet light (for 15 minutes), fertile eggs were then divided in five experimental groups: CF – non-injected eggs; CV – eggs injected with PD culture medium; F01 – eggs injected with *G. lucidum* MGF without elicitation; F02 – eggs injected with lignin-elicited *G. lucidum* MGF; and F03 – eggs injected with AS-elicited *G. lucidum* MGF. Each group had 40 eggs, totaling 200 incubated eggs. Each egg received 100 µL of the respective solution (at 20%) through the air chamber, using disposable syringes of 1 mL. After the injection, the eggshell orifice was sealed with adhesive tape and the eggs were kept in an incubator for three days, under a controlled temperature of 37.5 °C, relative humidity between 50% and 60% and constant forced ventilation.

After the incubation period, the eggs were opened to check the heart rate (HR), according to Kmecick (2017). Only embryos who presented heartbeats were used in other analyses. Eggs with no evidence of embryonic development were considered inviable and discarded. Taking into account that the chicken embryo's ability to feel pain begins to develop only from the seventh day of incubation (Rosenbruch 1997), there was no anesthetic protocol for euthanasia. Anyway, after the HR check, embryos were kept in a freezer, providing minimal stress to the animals (American Veterinary Medical Association 2020).

After euthanasia, the embryos were prepared using the whole mount technique as described by Ortolani-Machado et al. (2012). The morphological analysis occurred according to the descriptions of the stages of normal embryonic development for the species by Hamburger and Hamilton (1951) using a Stemi 305 stereoscopic microscope (Zeiss, Germany), equipped with an AxioCam ERc5s camera (Zeiss, Germany).

Statistical analysis

Embryotoxicity and teratogenicity test data were analyzed statistically using R, version 4.0.3 (R Core Team 2020) and RStudio, 1.3.1073 (Rstudio Team 2020), with the statistical packages *agricolae*, version 1.3-3 (Mendiburu 2020) and *lmtest*, version 0.9-38 (Zeileis and Hothorn 2002).

We used the generalized linear model, proposed by Nelder and Wedderburn (1972), to analyze data related to viability, survival, malformation occurrence and heart rate. Poisson distribution was applied to heart rate data and we used a logit-link binomial distribution to data concerning viability, survival and malformations rates. As a way to assess the goodness of fit, we used the Hosmer-Lemeshow test (Hosmer and Lemeshow 2000).

To compare the treatments, Wald test (Wald 1943) was performed for nested models and, when the result was significant ($p \leq 0.05$), Tukey's test (Tukey 1949) was applied at 5% of error probability. As so to analyze data referring to the embryonic stages of development, we used Kruskal and Wallis (1952) statistical approach, which is an extension of Wilcoxon-Mann-Whitney test for more than two groups (Neuhäuser 2011).

Results and discussion

Determination of minimum inhibitory concentration (MIC)

G. lucidum filtrates showed direct *in vitro* antimicrobial action on *E. diffusa* spores. Minimum inhibitory concentrations verified were 5 mg/mL for the *G. lucidum* MGF without elicitation and 10 mg/mL for elicited filtrates (Table 1). In contrast to what was expected, the addition of the elicitors in the submerged culture of *G. lucidum* did not result in a greater accumulation of antimicrobial compounds. In a previous study, we observed that *G. lucidum* filtrates could activate soybean defense mechanisms through a process called systemic acquired resistance, helping to control powdery mildew (Cruz et al. 2019).

Nonetheless, these results suggest the *G. lucidum* MGF potential in the control of plant disease, since most of the research related to fungi fruiting bodies and mycelium extracts only report the activity of isolated polysaccharides and manly just their antibacterial potential. Results on mycelial growth filtrates over phytopathogenic fungi are still limited, most of them *in vitro* studies. *G. lucidum* extracts have already shown bactericidal action, principally on *Bacillus cereus, Enterobacter aerogenes, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* and the diverse extraction methods (hexane, dichloromethane, ethyl acetate and methanol) offer different antimicrobial spectra against microorganisms strains (Kamra and Bhatt 2012).

Heleno *et al*. (2013) reported that *G. lucidum* methanolic extract showed greater activity against *S. aureus* and *B. cereus* than antibiotics ampicillin and streptomycin. Minimum inhibitory concentration were in the range

Table 1. *In vitro* minimum inhibitory concentration of *G. lucidum* MGF on *E. diffusa* spores by the microdilution broth method.

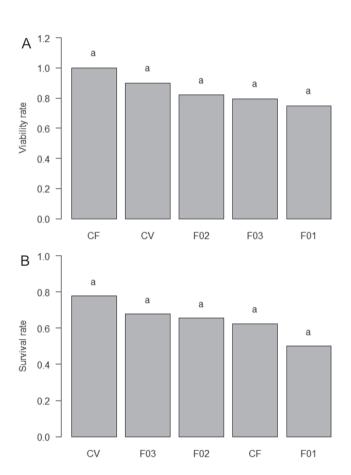| Treatments	MIC (mg/mL)
Distilled water	-
AS (2 mM)	-
Lignin (5%)	-
G. lucidum MGF (20%)	5
G. lucidum MGF + lignin (20%)	10
G. lucidum MGF + AS (20%)	10

Table 2. Number of embryos used for each analysis according to the amount of incubated eggs (IE), inviable eggs (IN) and dead embryos (DE) per experimental group.

Experimental group	Parameters analyzed						
	IE	IN	DE	S**	HR	M***	ES
Negative control (CF)	40	0	15	40	25	20	20
PD culture medium (CV)	40	4	8	36	28	23	20****
G. lucidum MGF (F01)	40	10	15	30	15	12	12
Lignin-elicited *G. lucidum* MGF (F02)	40	7	11	32*	21	17	17
AS-elicited *G. lucidum* MGF (F03)	40	8	10	31*	21	16	16

S: survival rate; HR: embryonic mean heart rate; M: morphological analysis; ES: embryonic staging.
* One embryo from each group was not considered for any of the analyses due to a manipulation error and consequent samples loss.
** Inviable eggs were disregarded for survival analysis.
*** Some embryos were not used in this analysis because they were not in good condition; CF, CV and F03 - 5 embryos; F02 - 4 embryos; and F01 - 3 embryos.
**** It was not possible to determine the stage of development of three embryos; two of them with severe malformations.

Figure 1. Egg viability (A) and survival rate (B) of *G. gallus* embryos exposed to *G. lucidum* mycelial growth filtrates (MGF) through the air chamber.

CF: negative control; CV: PD culture medium; F01: *G. lucidum* MGF; F02: lignin-elicited *G. lucidum* MGF; F03: AS-elicited *G. lucidum* MGF.
Means followed by the same letter do not differ statistically by Tukey's test, at 5% significance level.

of 0.0125 to 0.75 mg/mL and bactericidal concentrations from 0.035 to 1.5 mg/mL. Keypour et al. (2008) reported that the chloroform extract isolated from *G. lucidum* fruiting body inhibited *S. aureus* (8 mg/mL) and *Bacillus subtillis* (16 mg/mL). The study also found that a variety of lipid derivatives, like sterols and triterpenoid acids, were present in the extract.

Ćilerdžić et al. (2016a) were some of the few researchers who studied the antimicrobial and antioxidant activity of fermentation broth filtrates of *G. lucidum*. These authors report that the filtrates showed great antibacterial potential, inhibiting the growth of *S. aureus* and *E. coli* at concentrations of 6.25% and 12.5%, respectively, and against *Aspergillus niger*, *Penicillium cyclopium* and *P. aeruginosa* only at maximum concentration (100%). In this way, filtrates are potent antimicrobial agents and can be obtained more quickly and cheaply, when compared to *G. lucidum* fruiting bodies extracts (Ćilerdžić et al. 2016b).

Works with the isolation of proteins and polysaccharides have also been mentioned in the literature. An antifungal protein called ganodermin, isolated from *G. lucidum* fruiting body inhibited the growth of *Botrytis cinerea*, *Fusarium oxysporum* and *Physalospora piricola*, with an average inhibitory concentration value of 15.2 mM, 12.4 mM and 18.1 mM, respectively (Wang and Ng 2006). Two hydroquinones, ganomycins A and B, isolated from *Ganoderma pfeifferi* were effective for bacterial inhibition. MIC values of the compounds were 25 µg/mL against *S. aureus* and 2.5 µg/mL against *Micrococcus flavus* (Mothana et al. 2000).

The results of this work demonstrate that the filtrates of *G. lucidum* have the potential to be used as an agent of biological control against plant diseases. It is suggested that future studies focus on *in vivo* tests with plants, against other pathogenic microorganisms, and on the extraction of compounds that present antimicrobial properties. A better understanding of these compounds is crucial to identify

Table 3. Number of embryos considered to be normal (N) and malformed (MF) during the morphological analysis per experimental group.

Experimental group	N	MF
Negative control (CF)	20	-
PD culture medium (CV)	21	2
G. lucidum MGF (F01)	10	2
Lignin-elicited *G. lucidum* MGF (F02)	14	3
AS-elicited *G. lucidum* MGF (F03)	16	-

Malformation occurrence among the groups did not differ statistically by Tukey's test, at 5% significance level.

the potential effects on various diseases in the field.

Analysis of the effects on the early embryonic development of G. gallus

Table 2 shows the number of embryos used in each analysis according to the amount of eggs incubated, inviable eggs and dead embryos per experimental group. Both viability and survival parameters did not show significant differences when comparing data from treatments and control groups. Thus, it can be said that the *G. lucidum* MGF did not significantly affect egg viability and were not toxic to the development of the embryos of *G. gallus* (Fig. 1).

Since eggs did not complete total period of incubation, we are considering the viability parameter as the ratio between the number of eggs with embryos that resumed development out of the total number of incubated fertile eggs. Therefore, when opened, eggs both with alive and dead embryos were considered viable. Fig. 1A shows that *G. lucidum* MGF exposure did not significantly alter this variable. Viability rates among groups ranged from 75% in the F01 group to 100% in the CF group. In embryotoxicity studies, this parameter can indicate whether the tested substance has the potential to interfere with the resumption of normal embryo development, which ceases almost completely after laying, at temperatures below 25 °C (Bellairs and Osmond 2014). In the event of interference, the treated groups are likely to have very high unviability rates when compared to a control group. This type of analysis is uncommon and often disregarded in embryotoxicity studies, which preferentially report just mortality/survival rates among the groups. In this situation, the discussion is focused just on the number of dead/alive embryos out of the total eggs incubated, disregarding possible effects over development restart.

Fig. 1B shows the survival rate of *G. gallus* embryos exposed to the *G. lucidum* filtrates. Even though there is a numerical difference among the groups, the filtrates cannot be considered toxic or lethal to chicken embryos. Lee et al. (2003), when administering aqueous extract of *G. lucidum* intraperitoneally or orally, observed a significant increase in the survival of mice implanted with different tumors. Çelik and Özparlak (2019) investigated the possible genotoxic effects of the aqueous extract of wild *G. lucidum* using a type of micronucleus test on chicken embryo cells. Their results showed that the extract had no genotoxic action and, in addition, they have observed antigenotoxic properties. Similar works involving *G. lucidum* and avian embryos are scarce. Dulay et al. (2012) carried out perhaps the most significant study analyzing the direct effects of *G. lucidum* extracts on embryos. They observed significantly higher mortality rates, 72 hours after exposure, in groups of *Danio rerio* embryos treated with *G. lucidum* extract at 5%, 10% and 20%, and the lethal effect was dependent on dose and time of exposure.

Some authors report toxicity test results involving *G. lucidum* and other animals, adults in general. Atoji-Henrique (2015), using the same *G. lucidum* strain of our study, demonstrated that the supply of mycelium included in rabbit food did not interfere with the ingestive behavior, nor with the performance and other parameters of animal carcass. In the lowest of the concentrations (0.5%), intestinal segments related to the absorption of nutrients were favored. Nascimento et al. (2015) performed an acute test on Swiss mice and concluded that the administration of *G. lucidum* hydroethanolic extract (1 mL/kg) did not present significant toxicity.

As well as viability and survival rates, malformations occurrence did not vary significantly among groups (Table 3), which means that *G. lucidum* filtrates cannot be considered teratogenic to *G. gallus* embryos. These results are similar to those found by Özparlak et al. (2018), who, when analyzing the effects of wild and cultivated forms of *G. lucidum* on embryos of domestic chicken did not find embryotoxic or teratogenic effects, nor any interference in the bone development of embryos at macroscopic level. In the study by Dulay et al. (2012), performed with *D. rerio* embryos, it was possible to observe caudal malformations and growth retardation. Tail malformations were observed in 55.56% of embryos treated with 1% *G. lucidum* extract and in all embryos treated with the same extract at 5%. In the same way it was possible to verify a delay in the growth of embryos exposed to 5%, 10% and 20% extracts (Dulay et al. 2012).

During the morphological analysis, aspects of encephalic, optical and auditory vesicles, of limbs, neural tube and body curvature of the embryos were observed. The main malformations observed in the embryos, regardless of the experimental group, are shown in Fig. 2. Among the most representative are (i) gastroschisis (Fig. 2B and 2C); (ii) caudal atrophy (Fig. 2D); (iii) failure in telencephalon development (Fig. 2F); (iv) total malformation (Fig. 2E) – Fig. 2A shows a normal embryo. It is noteworthy that
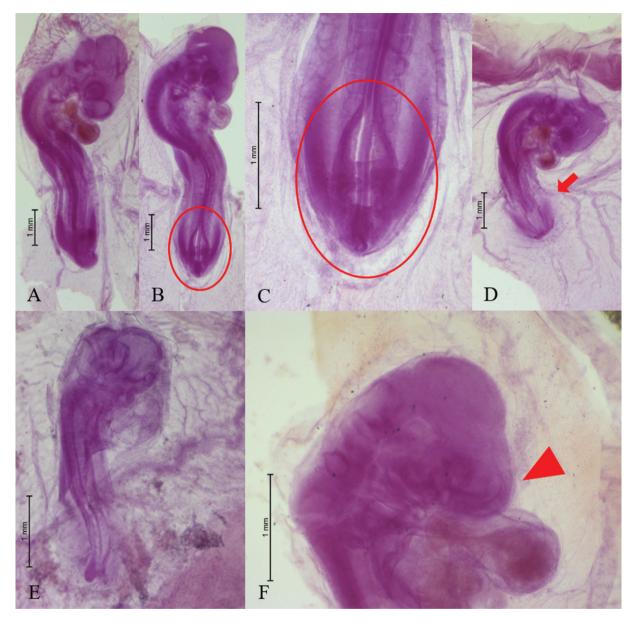
Figure 2. *G. gallus* embryos exposed to *G. lucidum* mycelial growth filtrates (MGF) through the air chamber and incubated for three days.

A: HH18 embryo with normal morphological traits; B: HH17 embryo presenting gastroschisis, shown in detail in C (circle); D: HH18 embryo with caudal atrophy (arrow); E: embryo in an undetermined stage of development, totally malformed; F: HH17 embryo showing failure in the telencephalon development (arrowhead).

congenital abnormalities have intrinsic and extrinsic causes and can even develop in normal environments (Gilbert and Barresi 2016; Carlson 2019). In consequence, errors are considered inherent to embryonic development.

Studies evaluating the toxicity and/or teratogenicity of fungal mycelial growth filtrates are insufficient. However, many studies report the effects of mycotoxins during embryonic development (Elsayed et al. 2019; Huang et al. 2019; Wu et al. 2019). Zahoor-ul-Hassan et al. (2012)

concluded that chicks and chicken embryos exposed to ochratoxin A, mycotoxin produced by fungi of the genera *Aspergillus* and *Penicillium*, presented significant anato-morphological alterations, congenital abnormalities and altered biochemical parameters. Saleemi et al. (2015) found similar results in tests with aflatoxigenic fungal isolates, especially aflatoxin B_1, in chicken embryos. Authors observed toxic effects and prominent embryo mortality rates in the groups exposed to the highest concentrations
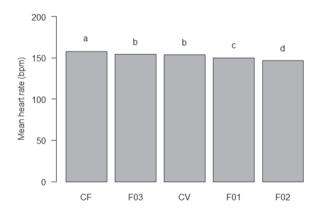
Table 4. Number of embryos per Hamburger-Hamilton embryonic stage and mean ranks (MR) per experimental group.

Experimental group	13	15	16	17	18	19	20	MR*
Negative control (CF)	-	1	2	2	9	6	-	51,1250
PD culture medium (CV)	-	-	2	9	5	3	1	41,8250
G. lucidum MGF (F01)	-	-	3	7	2	-	-	26,0417
Lignin-elicited *G. lucidum* MGF (F02)	1	-	1	6	5	3	1	44,2941
AS-elicited *G. lucidum* MGF (F03)	-	-	-	7	7	1	1	45,6563

* Mean ranks assigned by the Kruskal-Wallis test.

of aflatoxins (up to 100 ng/egg).

Regarding heart rate, there was a significant decrease when *G. lucidum* MGF (F01) and *G. lucidum* + lignin (F02) were applied. CV and F03 groups also showed a significant difference compared to the CF group, but without differences between themselves (Fig. 3). Average heart rate for the CF group remained at 157.8 bpm. The result is close to the average value of 150 bpm found by Akiyama et al. (1999), despite the difference regarding the methods, and the value of 153 bpm verified in embryos of 72 hours in the control group by Kmecick (2017). Ritchie et al. (2013) state that drugs that can induce periods of bradycardia can be potential teratogens for humans.

Through morphological analysis, it was possible to identify seven different Hamburger-Hamilton (HH) embryonic stages (HH13, HH15–HH20), predominantly stages HH17 and HH18 (Table 4). As the parameter "developmental stage" is a qualitative variable (not numerical), by the analysis using the Kruskal-Wallis test it was possible to verify a significant difference among the

stages of development of F01 embryos when compared with embryos of CF, F02 and F03 groups, which did not present statistically significant differences among them. Mean number of ranks for the CV group was statistically equal to that of the other groups (Fig. 4).

This means that embryos exposed to *G. lucidum* MGF without elicitation were, at the time of collection, in younger stages of development than embryos from the other experimental groups. Statistically significant reduction in mean heart rate, observed in the F01 group (Fig. 3), may have limited cardiac output (Branum et al. 2013), which may have resulted in a delay in embryonic development. This factor, however, does not seem to have affected the other groups that also had a lower average heart rate, such as F02 and F03.

Studies involving hypothermia, reduced heart rate and delayed normal embryonic development corroborate this hypothesis. Burggren et al. (2016) consider temperature as a disruptive agent in the normal development process, since low temperatures can delay development (Tazawa

Figure 3. Mean heart rate (HR) of *G. gallus* embryos exposed to *G. lucidum* mycelial growth filtrates (MGF) through the air chamber and incubated for three days.
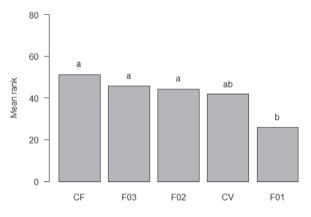
CF: negative control; CV: PD culture medium; F01: *G. lucidum* MGF; F02: lignin-elicited *G. lucidum* MGF; F03: AS-elicited *G. lucidum* MGF.
Means followed by different letters were considered statistically different from each other by Tukey's test, at 5% significance level.

Figure 4. Comparison of the average number of ranks distributed by the Kruskal-Wallis test regarding Hamburger-Hamilton (HH) embryonic stages.

CF: negative control; CV: PD culture medium; F01: *G. lucidum* MGF; F02: lignin-elicited *G. lucidum* MGF; F03: AS-elicited *G. lucidum* MGF.
Means followed by different letters were considered statistically different from each other by the Kruskal-Wallis test, at 5% significance level.

1973) and growth (Peterka et al. 1996). In addition, Lee et al. (2011) observed that environmental hypothermia could reduce the normal heart rate of young chicken embryos (HH17 stage). Kockova et al. (2013) concluded that periods of bradycardia could lead to embryonic death by decreasing cardiac output, since the heart of embryos is still unable to adjust stroke volume according to variations in heart rate. Branum et al. (2013) could not observe a delay in the rate of development when exposing chicken embryos to a bradycardic drug – which decreased cardiac frequency and output. However, these authors were able to notice a 20% reduction in the growth rate, measured by the embryos' body mass.

Studies emphasizing the interference relationships of biocompounds on the stages of embryonic development are scant. Recently, in a test very similar to ours, Vismara (2019) did not observe statistically significant differences regarding the developmental stages of *G. gallus* embryos treated with essential oils of plant species. Arcain (2017) found a similar result, noticing no differences among control and treated groups, regarding stage of development, when exposing *G. gallus* embryos to a commercial synthetic fungicide.

Conclusion

We have concluded that *G. lucidum* filtrates showed fungicidal action on *E. diffusa in vitro* with a minimum inhibitory concentration of 5 mg/mL. Therefore, the MGF of *G. lucidum* shows potential to be used to control powdery mildew in soybean. New studies seeking to verify the *G. lucidum* MGF potential in other pathosystems of agricultural interest should be considered.

It was also possible to conclude that *G. lucidum* filtrates did not affect the embryonic development of *G. gallus*, proving to be non-toxic to this non-target organism. Despite this, significant differences were observed in the mean heart rate and stage of embryonic development, indicating a potential bradycardic effect of *G. lucidum* filtrates without elicitation. Our results corroborate the understanding that this model organism is ideal for embryotoxicity and teratogenicity studies, as pointed out by previous works. New tests are recommended to identify, isolate and characterize molecules present in the filtrates, so that the mechanisms of action on the embryonic development can be better understood.

Acknowledgements

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