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Bioactive Compounds of *Ganoderma lucidum* Activate the Defense Mechanisms of Soybean Plants and Reduce the Severity of Powdery Mildew

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Abstract

Ganoderma lucidum is a medicinal mushroom widely used in Eastern countries and currently in global scale. Its fruiting body and mycelium are composed by polysaccharides, triterpenes and more than 200 secondary metabolites. These compounds exhibit a range of bioactivities, such as anti-inflammatory, antitumorigenic, antibacterial and antifungal action. Several scientific publications have demonstrated the potential and performance of *G. lucidum* compounds in the control of diseases in animals and humans. However, there is a lack of information on the effect of their compounds on the phytopathogens control, whether directly or by activating plant defense mechanisms. In the search of new molecules that has induced activity and disease control, this study was aimed to evaluate the bioactive compounds produced by *G. lucidum* through liquid culture under elicitation to control powdery mildew (*Erysiphe diffusa*) in soybean plants. The compounds tested were: C01-distilled water, C02-copper oxychloride (1 L ha^{-1}), FC01-filtered mycelial growth of *G. lucidum* without elicitation, FC02-*G. lucidum* filtration of lignin elicitation and FC03-*G. lucidum* filtration from SA elicitation. The upper part of the plant was sprayed at 20 % (v/v) concentration and 10 mL per plant were applied. All data were analyzed using R® software. The *Ganoderma* filtrates have shown the induction of resistance potential in soybean plants by the activation of phytoalexins, activation of the enzyme phenylalanine ammonia-lyase, increase of phenolic compounds, peroxidases and chitinase activity, such induction has specificity in relation to time activation and association with elicitors. New studies should be considered, seeking to identify and isolate the active principles present in the filtrates, as well as to evaluate the action of these substances in other pathosystems of agricultural interest.

Keywords: medicinal fungi, submerged culture, bioprospecting, alternative control, elicitation

1. Introduction

Brazil is among the largest producers of soybeans (*Glycine max* (L.) Merrill) in the world. In 2017/2018, according to the report of the National Company of Supply (CONAB) the country produced 119.28 million tons of grain (CONAB, 2018). The occurrence of diseases is one of the limiting factors for increased soybean yield and crop sustainability due to losses and problems related to the abuse of agrochemicals (Godoy et al., 2016).

Among these diseases, powdery mildew [*Erysiphe diffusa* (Cooke & Peck) U. Braun & S. Takamatsu] is important by the significant loss in soybean crops (De Almeida; Forcelini, Fiallos, 2017). This fungus develops on the surface of the leaf, forming a thin layer of mycelium and reducing the active photosynthetic area. This leads to the drying of leaves and premature fall, causing yield losses from 10% to 50%, hindering the development of the plant (McTaggart et al., 2012).

Fungicides are considered the main control method used in the management of diseases, for presenting practicality and efficiency. However, intensive and erroneous applications have brought negative consequences, such as loss of effectiveness, selection of resistant phytopathogenic agents, emergence of iatrogenic diseases

(those that occurred due to the use of agrochemicals) and contamination of food and soil (Klosowski et al., 2016). Furthermore, public demand for environmentally safer methods of control drives the industry and producers to seek other alternatives.

Over recent years, alternative methods have been intensely studied and employed in modern agriculture. The use of biological control agents (biocontrol), biostimulants, biopesticides, biofungicides and the induction of resistance of plants against pathogens are increasing strategies to control plant diseases, seen as promising alternatives to the use of commercial fungicides (Bettiol, 2008).

The induction of resistance involves the activation of latent defense mechanisms in plants in response to the treatment with biotic or abiotic agents, called elicitors (Smith, 1996). Activation of plant defenses may occur from the elicitation with the use of living organisms such as bacteria, fungi, viruses, nematodes and insects—including non-virulent pathogen strains—beyond the inactivated pathogen itself, plant extracts, growth promoting rhizobacteria, and even bacterial and fungal exopolysaccharides (Stadnik & Bettiol, 2000). Currently, there are commercially available resistance inducers, which have not come to replace traditional fungicides, but their use in conjunction or alternating may lead to a reduction in the number of pesticides applications (Di Piero et al., 2003; Kuhn, 2007).

Biofungicides are composed of microorganisms such as fungi or bacteria, with a direct effect on the pathogen; or indirect activating resistance genes (Harman, 2000). These microorganisms act by secreting chemical compounds with antibiotic and antifungal action, such as enzymes and secondary metabolites, which leads to the success of the use of these fungi as biofungicides, since several of these secondary metabolites produced are toxic to plant pathogens even in low concentrations (Morath et al., 2012).

Among the agents with the potential to produce bioactive substances and biotic resistance inducers, there is G*anoderma lucidum*, a medicinal mushroom traditionally used in Eastern medicine over a few thousand years. Known as “Lingzhi” or “Reishi” in East Asia and as “Cogumelo Rei” in Brazil (Cao et al., 2012), this mushroom synthesizes antibiotics, immunomodulators, antiallergics and many other compounds of medicinal interest (Lindequist et al., 2005; Boh et al., 2007).

Substances that provide therapeutic characteristics from this mushroom are called bioactive compounds, and include polysaccharides, oligosaccharides, triterpenoids, peptides, proteins, alcohols, phenols, mineral elements, vitamins and amino acids (Batra et al., 2013). The mycelium of *G. lucidum* presents the same bioactive compounds of the fruiting body in similar concentrations, and the production time is shorter, ranging from 7 to 30 days (Liu et al., 2012). Many researchers seek the production of therapeutic compounds in liquid medium, testing different culture media and forms of elicitation. At times, the cultivation in liquid medium becomes the fastest and most efficient process, leading to the production of target substances (Zhang, Zhong, & Geng, 2014; Liu, 2018).

G. lucidum mycelium is a source of several substances, which can be explored for the management of plant diseases. The use and study of medicinal and/or edible mushrooms in the control of plant diseases are relatively recent and are mainly concentrated in the *Lentinula edodes* (shiitake), *Agaricus blazei* (= *A. subrufescens*) (sun mushroom) and *Pycnoporus sanguineus* (royal sun mushroom) species (Schwan-Estrada et al., 2017).

Researches with *G. lucidum* on plants are still minimal. However, there are innumerable studies about the effects of *G. lucidum* compounds on disease control in animals and humans. Its characteristics reveal a potential for investigation of elicitor molecules with eventual use in the activation of defense mechanisms in plants. Moreover, the absence of studies, in the literature, related to resistance inducers obtained from fungal biomass of *G. lucidum* justifies the importance of the present study. This study aimed to obtain bioactive compounds produced in the liquid medium culture of *G. lucidum* and evaluate the potential of these substances on the control of powdery mildew in soybean

2. Method

2.1 Organism

Ganoderma lucidum CC339ST was obtained from Brazilian Agricultural Research Corporation-Genetic Resources and Biotechnology (EMBRAPA CENARGEN). Cultures were inoculated on Potato Dextrose Agar (PDA) Petri dishes and incubated at 28 °C for 7 days. Fully colonized Petri dishes were stored at 4 °C. Mycelium was activated on PDA with the same procedure and then used for inoculation in liquid medium. The isolate of *Erysiphe diffusa* (Cooke & Peck) U. Braun & S. Takamatsu] was maintained on soybean seedlings (cultivar NA5909).

2.2 Submerged Fermentation

For the fermentation process, 0.5 cm^2 discs of myceliated agar were aseptically transferred to 500 mL Erlenmeyer's containing 250 mL of PD (potato-dextrose) liquid medium. The inoculated flasks were initially incubated in rotary shaker (120 rpm) in the dark at 28±2 °C, for 15 days.

After this period, the elicitors were added to the fermented medium and those were salicylic acid (SA) at 2 mM concentration, 5% lignin standard. Salicylic acid and lignin were dissolved in water, sterilized and subsequently added to Erlenmeyer's with *G. lucidum*.

The Erlenmeyer's containing the treatments with the elicitors were kept in an orbital shaker (120 rpm) in the dark at 28±2 °C, by further 35 days, totaling 50 days of fermentation in liquid culture. After this period, the media were filtered on Whatman paper no. 41 for mycelium separation, obtaining the mycelial growth filtrate (MGF). The treatments were: FC01-mycelial growth filtrate of *G. lucidum* without elicitation; FC02-lignin elicitation filtrate; FC03- SA elicitation filtrate.

2.3 *Activation of Phytoalexins in Soybean Cotyledons*

Soybean seeds were sown in polypropylene trays containing autoclaved sand and kept in a BOD chamber at 25 °C with a 12 hour of photoperiod. After 10 days, the cotyledons were removed and washed in distilled water. On the abaxial side of the cotyledons was made a superficial cut and over the cut were deposited 40 µL of the treatment solutions, consisting of FC01-mycelial growth filtrate of *G. lucidum* without elicitation; FC02-filtration of elicitation with lignin and FC03-filtration of elicitation with SA. The controls were: C01-distilled water and C02-copper oxychloride (1 L ha^{-1}). The MGF were used at concentrations of 2%, 5%, 10% and 20% (v/v). The cotyledons were weighed and placed in Petri dishes (four cotyledons per plate) covered with filter paper disc dampened with distilled water. Covered and unsealed plates were maintained in BOD at 26 °C in the dark. After 20 hours, the cotyledons were removed from the plates and placed in plastic tubes containing 10 mL of distilled water, which were agitated on a shaker table at 80 rpm for one hour, for the extraction of glycerol. After that, the solution was filtered on Whatman filter paper no. 41 and the absorbance was determined by spectrophotometer at 285 nm wavelength, according to the methodology described by Labanca (2002).

The experimental design was completely randomized, comprising fifteen treatments with five replicates. The results were submitted to analysis of variance (ANOVA), where the assumptions of normality and homogeneity of the errors were evaluated through the tests of Shapiro-Wilk and Bartlett, respectively. The averages of the treatments were analyzed and compared by the Scott Knott test ($p < 0.5$) through the RStudio® Software with ExpDes.pt packages.

2.4 Protection of Soybean Plants Under Greenhouse Conditions

Soybean seeds of cultivar BRS 284 were sown in polyethylene pots with 10 liters capacity, containing soil from soybean crop. The pots were maintained in a greenhouse at an average temperature of 25±2 ºC until V6 stage (beginning of flowering).

Soybean plants were sprayed with 20% (v/v) MGF aqueous solution using a CO_2 pressurized sprayer at 150 kPa to 5 mL volume per pot. Subsequently to the application the pots were sorted randomly in the greenhouse. After 24 hours of treatments, the plants were inoculated with the pathogen, and plants containing symptoms of powdery mildew were arranged in greenhouse with healthy plants to have a contact with *Erysiphe diffusa* spores.

For the enzymatic analyzes, leaf segments (approximately 1 g of fresh tissue) were collected in pots containing five plants (repetition), in intervals of 0, 24, 48, 96 and 168 hours after MGF application. After the collection, samples of plant tissue were immediately stored in liquid nitrogen. These samples were used to quantify total proteins, phenolic compounds, activity of peroxidase enzymes, phenylalanine ammonia-lyase (PAL), chitinase and β-1.3 glucanase. A split plot experimental design by time was used, with five replications. Both treatments were considered as qualitative and, therefore, submitted to average comparison tests.

2.5 Enzyme Extraction

The frozen leaf segments were homogenized in 4 mL of 100 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA and 150 mg polyvinylpolypyrrolidone. The extract was centrifuged and homogenized (Biovera RB1-R microprocessed bench centrifuge) at 20.000 rpm min^{-1} for 30 min at 4 ^oC, considering the supernatant obtained as an enzymatic extract.

Protein contents were determined by Bradford test (1976) with 0.2 molar phosphate buffer, pH 7.5 and Bio-Rad® reagent. The readings were performed with spectrophotometer (630 nm); bovine serum albumin was the standard.

The determination of PAL was performed by the colorimetric quantification of trans-cinnamic acid of phenylalanine, according to Kuhn (2007). The extraction of peroxidase and determination of activity were performed according to Matsuno and Uritani (1972), in test tube was added 5 mL of citrate buffer solution (pH 5.0), 0.5 mL of hydrogen peroxide at 3%, 0.5 mL of 0.5% guaiacol and 3.0 mL enzyme extract. The readings were performed by spectrophotometer at a 450 nm wavelength.

The quantification of total phenolic compounds was carried out in two steps, following the method adapted from Bieleski and Turner (1966). The first one encompassed the extraction of total phenols made from the addition of methanol, chloroform and water (MCW) solution. Subsequently, the second stage encompassed the determination of total phenols performed by the method adapted from Jennings (1991). Absorbance readings were performed at 760 nm wavelength by spectrophotometer.

For the quantification of chitinase and β-1.3 glucanase activities, the samples were macerated in 4.0 mL of 100 mM acetate buffer (pH 5.0), with subsequent centrifugation (20.000 g for 25 min at -4 °C). The supernatant was collected and used for the evaluation of enzyme activity. The enzymatic activity of chitinase was evaluated by the release of "CM-chitin-RBV" soluble fragments from carboxymethylated chitin marked with bright violet remazol. The absorbance of the supernatant at 550 nm was determined as reference and the results were expressed in units of absorbance.min^{-1} (mg of protein)$^{-1}$, discounting the control absorbance values (800 μL of extraction buffer + 200 μL of "CM-chitinRBV"). For the spectrophotometric determination of β-1.3 glucanase activities, bright blue carboxymethylcurdlan-remazol solution (CM-Curdlan-RBB 4 mg/mL, Loewe Biochemica GmbH) was used as a substrate in the extracts according to the methodology developed by Wirth & Wolf (1992) and with the procedure described by Guzzo and Martins (1996).

The results were submitted to analysis of variance (ANOVA). The assumptions of normality and homogeneity of the errors were evaluated through the Shapiro-Wilk and Bartlett tests, respectively. The averages of the treatments were analyzed and compared by Tukey test, given a significance of 5%. All statistical tests were calculated using R® Software and the ExpDes.pt package.

2.6 Evaluation of Disease

The severity evaluation began when the first symptoms appeared in the plants. The evaluation was carried out according to a diagrammatic scale proposed by Matiazzi (2003). Five evaluations were performed, with 10 days intervals. For each data set obtained, the area under the disease progress curve (AUDPC) was calculated using the following equation (Shaner & Finney, 1977):

$$\text{AUDPC} = \sum_{i}^{n-1}[(y_i + y_{i+1}) \times 0.5]\cdot[t_{i+1} - t_i] \quad (1)$$

Where, n = total number of observations; y_i = intensity of disease at the i-th observation; t_i = time in the moment of i-th observation.

The data were submitted to the Lilliefors Normality test to verify compliance with the assumptions of the mathematical model. These data had not normal distribution and were therefore transformed using the square root operation. The averages of the treatments were submitted to the Tukey test, level of 5% of error probability. The statistical tests were calculated using the Software R®.

3. Results

3.1 Phytoalexin Assays in Soybeans

All treatments with MGF had increased the expression of the phytoalexin activity in soybean in relation to the control (Table 1). The filtrate of *G. lucidum* submitted to SA elicitation at concentrations of 10% and 20% were the treatments that induced the greatest accumulation; the filtrate from the 20% lignin elicitation also demonstrated similar effect.

The accumulation of glyceollin was observed from 5% concentration of all the filtrates when compared to the controls. The expression of defense responses was related to the dose of the elicitors. In other words, a higher accumulation of phytoalexins occurred according to increasing concentrations of the filtrates. The copper oxychloride compound had no effect on the induction of glyceollin in soybean.

The plants, like other organisms, have metabolic pathways that remain in silent until be activated. Secondary plant metabolites contribute to preinvasive resistance, disrupting the potentially invasive growth of fungal parasites (Smith, 1996).

Phytoalexins accumulate in cells near the fungus penetration point and act on the fungus through cytoplasmic granulation, disorganization of cellular contents, rupture of the plasma membrane and inhibition of fungal enzymes, reflecting on the germination and the elongation of the germinative tube inhibition and reducing or inhibiting the mycelial growth (Cavalcanti et al., 2005; Smith, 1996).

Many phytoalexins reported represent phenylpropanoids derived from phenylalanine. Several types of Fabaceae isoflavonoids constitute a phytoalexin group derived from phenylalanine. These include, among many others, glyceollin synthesized by soybean (*Glycine max*) (Ebel et al., 1976).

In the prior literature, there are no reports about the use of *G. lucidum* as elicitor of defense responses in vegetables. In the present study was verified the efficiency of the *G. lucidum* filtrates as a source of elicitor molecules for the synthesis of glyceollin in soybean plants.

Table 1. Induction of phytoalexin glyceollin in soybean cotyledons by *G. lucidum* MGF submitted to different elicitation treatments

Treatments	Phytoalexines Abs (285nm/g.p.f.)
Ganoderma +SA 20%	0.285 a
Ganoderma +SA 10%	0.269 a
Ganoderma + Lignin 20%	0.237 a
Ganoderma + SA 5%	0.221 b
Ganoderma + Lignin10%	0.218 b
Ganoderma + Lignin 5%	0.208 b
Ganoderma 10%	0.199 b
Ganoderma 20%	0.198 b
Ganoderma 5%	0.165 b
Ganoderma + Lignin 2%	0.104 c
Ganoderma + SA 2%	0.092 c
Copper oxychloride	0.087 c
Ganoderma 2%	0.054 c
Distilled water	0.047 c
CV = 19.32 %	

Note. Averages followed by the same letter in the column did not differ significantly by the Scott Knott test ($p < 0.5$).

The elicitor treatment leads the plant to a “primming” state of alert, which makes the defense response faster and intense at the moment of contact with the pathogen (Conrath et al., 2006). According to Fauth et al. (1996), the injury caused in this type of bioassay is sufficient to condition cotyledonary tissues, and subsequent treatment with the inducer plays the role of the pathogen, leading to the synthesis of defense compounds. Thus, the treatment of soybean cotyledons with the MGF causes phytoalexins synthesis without the need of contact with the pathogen.

A similar study was carried out by De Arruda et al. (2012), who demonstrated the extracts of mushrooms Agaricus blazei (= *A. subrufescens*), *Lentinula edodes* and *Pycnoporus sanguineus* at 20% (v/v) concentration show an effect on Erysiphe diffusa and also induction of resistance in the activation of glyceollin. Fiori-Tutida (2003) attained results on the induction of phytoalexins in soybean cotyledons and sorghum mesocotyls, using different isolates of *A. blazei* (= *A. subrufescens*) and *L. edodes*. Beninca (2007) observed the extraction with dichloromethane, hexane and ethanol of *P. sanguineus* basidiocarpos did not activate the synthesis of phytoalexin glyceollin, but the synthesis of deoxyanthyanidines in sorghum.

In studies conducted by Piccinin (2000), *L. edodes* preparations had induced the phytoalexins production of the deoxythyanocyanine complex in sorghum mesocotyls, and glyceollin in soybean cotyledons. In the same way, the filtrates of mycelial growth and mushroom strain were efficient in reducing local and systemic infections by *X. axonopodis* pv. *Passiflorae in passion* fruit.

Mycelium of *G. lucidum* was applied to peanut callus in comparison with a peanut pathogen *Botryodiplodia theobromae* to understand the effect of Reishi on stilbenoid elicitation. Results showed the amounts of piceatannol and resveratrol induced by Reishi were comparable to those induced by the pathogenic one. *Ganoderma lucidum* is a potent elicitor in production of phytoalexins t-Resveratrol and t-Piceatannol - the

well-known health-promoting active components in plants are secondary metabolites generated upon biotic or abiotic stresses (Yang et al., 2010).

On the basis of achieved results of this study, it can be stated that the filtrates of *G. lucidum* have the potential to induce the synthesis of phytoalexins in cotyledons of soybean, presenting vegetal defence specificity of the activation in function of the products concentrations used in the elicitation.

3.2 Activity of Total Proteins in Soybean Plants

In the experiment carried out in the soybean pathosystem/powdery mildew in greenhouse, according to the Table 2, it has been found a significant increase in the values of plants treated with MGF and inoculated with powdery mildew when compared to the control and the treatments with fungicides.

This significant increase in protein activity indicates that the filtrates have action on the primary metabolism of plants. Protein accumulation in soybean plants began 48 hours after treatment with the filtrate subjected to elicitation with SA, and remained growing until 192 hours after application. In general, all MGF have stimulated the protein concentration increase in soybean plants after 192 hours when compared to the control. There was no statistical difference between the treatments, in which only water was applied, and the plants that received fungicide application based on copper oxychloride.

Proteins are polymers of amino acids. These amino acids are precursors to many plant metabolites related to the defense. They also present structural and dynamic functions and participate in several biochemical reactions as enzymatic catalysts. Among the proteins, there are those related to pathogenesis (RP-Proteins). Proteins related to the pathogenesis are essential components of the plant defense response, its expression can be induced at the site of pathogen infection or systemically in the host (Van Loon et al., 2006).

Table 2. Total proteins activity (mg.g tissue^{-1}) of soybean plants treated with MGF of *G. lucidum* and copper oxychloride

Time (hours)	Treatments				
	Control	Gano	GanoSA	GanoLig	Copper oxychloride
0	1.79 aB	2.26 aB	1.83 aB	2.11 aAB	2.31 aA
48	2.00 aA	1.95 aB	2.17 aA	1.84 aB	1.93 aAB
96	1.52 bAB	2.21 abB	2.36 aA	2.43 aAB	1.62 bAB
192	1.99 bA	2.98 Aa	2.53 aA	2.80 aA	1.51 cB

**CV = 17.90%; ***CV = 19.59%

Note. Averages followed by the same lowercase horizontal letter, or upper case vertical, are not significantly different by Tukey's test, at the ($p \leq 0.05$) error probability level. The elicitors *G.lucidum* (Gano), *G.lucidum* + Salicylic Acid (GanoSa), *G.lucidum* + lignin (GanoLig), Copper oxychloride (1 L ha^{-1}), distilled water (control). * Treatments coefficient of selection. ** Collection dates coefficient of variation.

The induction of resistance in plants can be triggered by several elicitors, among them, the mycelial growth filtrates of *G. lucidum*. The compounds present in the filtrate had acted as elicitors that were liaised with the receptors on the plasma membrane of the plant cell. Afterwards, this sign is transmitted inside the cell, activating the messenger substances which amplify the sign and regulate specific genes expression, resulting in the activation of mechanisms of resistance to phytopathogens (De Arruda et al., 2012).

After detection of the pathogen, plants are able to induce a number of defense mechanisms, including closing of the stomas to limit the bacterial invasion (Melotto et al., 2008; Sawinski et al., 2013), production and secretion of antimicrobial compounds including phytoalexins as camalexin, proteins and peptides, such as PR1 (Cowan, 1999; Ahuja et al., 2012; Bednarek, 2012). Generation of reactive oxygen species (ROS), which have effects on pathogens (O'Brien et al., 2012), and programmed cell death (PCD), referred to as hypersensitive response, on site of infection to limit the progression of the pathogen (Mur et al., 2008).

Among the potential inducers of resistance there are edible and medicinal mushrooms. In a study contucted by Viecelli et al. (2009), it was observed that bean plants treated with culture filtrate of *Pycnoporus sanguineus* against *Pseudocercospora griseola*, there was an increase in the protein content. In a study testing the filtrates of *P. sanguineus* for activation of defense mechanisms in beans, the authors have shown that the levels of peroxidase, polyphenoloxidase and protein and chlorophyll content were higher in the treated plants (Viecelli et al., 2009).

3.3 Phenylalanine Ammonia-Lyase (PAL)

In relation to the activity of the phenylalanine ammonia-lyase enzyme in soybean plants, the results presented in Table 3 demonstrate that plants treated with the MGF have presented higher activity of the enzyme compared to untreated plants.

There was an expressive increase in the synthesis of this enzyme in 48 hours after applications. This rapid increase in treated and untreated plants can be related to the activation of the resistance induced by pathogen colonization, that occurs 24 hours after the treatment of the plants, which possibly served as elicitor. The increase in PAL activity after inoculation is a common reaction of the plant to powdery mildew and has been frequently related to some forms of resistance (Mayama & Shishiyama, 1978).

The activity of the enzyme PAL was pronounced after 192 hours of Ganoderma + SA application. For additional treatments, the values were lower and decreased over time. Thus, it is pertinent to assume the activation of this enzyme in the treated plants was later compared to the untreated plants. This suggests that *G. lucidum* MGFs are efficient elicitors for the enzyme phenylalanine ammonia-lyase synthesis in soybean plants.

Thus, the results obtained confirm that *G. lucidum* MGF has an action on the secondary metabolism of the plant activating the route of phenylpropanoids. Therefore, the MGF application in soybean plants leads to the expression of the PAL enzyme and subsequent increase the defense responses against the powdery mildew.

Table 3. Activity of the enzyme Phenylalanine Ammonia-Liase (PAL) (mg/g.tissue) in soybean plants treated with MGF of *G. lucidum* and copper oxychloride

Time (hours)	Treatments				
	Control	Gano	GanoSA	GanoLig	Copper oxychloride
0	0.0073 aB	0.0068 aB	0.0079 aC	0.0067 aC	0.0059 aB
48	0.0131aA	0.0139 aA	0.0138 aB	0.0120 aB	0.0140 aA
96	0.0104aAB	0.0101 aB	0.0143 aB	0.0144 aAB	0.0131 aA
192	0.0079 cB	0.0151 bA	0.0216 aA	0.0162 bA	0.0150 bA
**CV = 30.01%; ***CV = 18.61%					

Note. Averages followed by the same lowercase horizontal letter, or upper case vertical, are not significantly different by Tukey's test, at the ($p \leq 0.05$) error probability level. The elicitors *G. lucidum* (Gano), *G. lucidum* + Salicylic Acid (GanoSA), *G. lucidum* + lignin (GanoLig), Copper oxychloride (1 L ha^{-1}), distilled water (control). * Treatments coefficient of selection. ** Collection dates coefficient of variation.

The expressive increase of PAL synthesis at 48 hours after application may be related to the contact of the pathogen with the plant. The fungus, after inoculation in the plants, takes on average 6 to 10 hours to germinate, form the appressorium and colonize the plant tissue; host responses occur during this time, including some biochemical responses that may influence the result of the imminent encounter between the appressorium and the host cell. After four to five hours of inoculation, the activities of three key enzymes involved in plant secondary metabolism can be expressed. These are: phenylalanine ammonia lyase (PAL), tyrosine (TAL), peroxidase and phytoalexins (Green et al., 1975).

Phenylalanine ammonia-lyase is the first enzyme of phenylpropanoid metabolism in most plants, and has been referenced by its important role in controlling the accumulation of phenolic compounds in response to the infection. The SA biosynthesis, as well as most phenolic compounds in plants, depends on the phenylalanine biosynthesis that is synthesized from erythrose-4-phosphate and phosphoenolpyruvate, through a string of reactions that compose the Shikimate/Arogenate pathway (Strack, 1997). It is a extensively studied enzyme by virtue of its key importance in the plants primary and secondary metabolism.

In all metabolic routes intermediates are produced to feed other pathways. In the case of phenylpropanoids, the increase of PAL leads to an increase in the phenylalanine synthesis, which may lead to an increase in coumarate concentration. The coumarate can either continue in the phenylpropanoid pathway and lead to increase the lignin synthesis (associated with increased peroxidase activity), as well as be diverted to the production of hydroxybenzoates, flavonoids or other phenols (Labanca, 2002). In other words, the increase of the PAL enzyme will not necessarily always result in the increase of phenolic compounds and peroxidases.

Some studies correlate the application of mushroom compounds with the activation of PAL enzyme. Baldo (2008) observed the application of extracts of *P. sanguineus* in bean plants caused local and systemic increase in the

specific activity of phenylalanine ammonia-lyase. Although polysaccharides from *L. edodes* were efficient against bacterial blight, it did not activate the enzyme phenylalanine ammonia-lyase in tomato plants. However, the treatment with polysaccharides increased the activity of peroxidases and total phenol content (Aguiar, 2016).

Some of the non-enzymatic metabolites produced by plants, such as flavonoids, anthocyanins and phenols, are originated from the shikimic acid pathway. These compounds are important in plant defense metabolism, including non-enzymatic defense against oxidative stress (Carlini & Ligabue-Braun, 2016). This correlation was verified in this study, including the activation of the shikimic acid route and increase in the production of peroxidases and phenolic compounds.

3.4 Total Phenols

According to the results demonstrated in this study, the activation of defense responses was visualized through increases in PAL enzyme activity and also by the accumulation of total phenolic compounds in soybean plants.

The results for total phenols showed a similar tendency to those of PAL enzyme activation. According to Table 4, there was a higher content of total phenolic compounds in plants treated with Ganoderma + lignin filtrate at 48 hours after application and 24 hours after inoculation of the pathogen, when compared to untreated plants and other treatments.

On the other hand, after this peak activation, the phenol contents were reduced, and this reduction in phenol concentration may be related to the synthesis of peroxidases as a defense mechanism. According to Kuhn and Pascholati (2010), the reduction of phenolic compounds may have occurred primarily as far as the cells became lignified, since phenolic compounds are substrate for the synthesis of lignin by the action of peroxidases. Thus, the subsequent reduction in phenol content would be a consequence of the higher peroxidase content in the treated plants.

For the treatment with Ganoderma + SA filtrate, the results also resemble those found for PAL enzyme, obtaining a greater accumulation of phenols after 192 hours, while there was a reduction for the other treatments.

Table 4. Phenolic compounds (mg.g tissue^{-1}) from soybean plants treated with *G. lucidum* MGF and copper oxychloride

Time (hours)	Treatments				
	Control	Gano	GanoSA	GanoLig	Copper oxychloride
0	0.39 aB	0.41aB	0.37 aC	0.34 aC	0.33 aB
48	0.86 bA	1.02 bA	0.92 bB	1.37 aA	0.77 bA
96	0.88 aA	1.08 aA	0.94 aB	0.94 aB	0.77 aA
192	0.65 cB	1.07abA	1.26 aA	0.99 bB	0.91 bcA
*CV = 21.46%; ***CV = 21.62%					

Note. Averages followed by the same lowercase horizontal letter, or upper case vertical, are not significantly different by Tukey's test, at the ($p \leq 0.05$) error probability level. The elicitors *G. lucidum* (Gano), *G. lucidum* + Salicylic Acid (GanoSa), *G. lucidum* + lignin (GanoLig), Copper oxychloride (1 L ha^{-1}), distilled water (control). * Treatments coefficient of selection. ** Collection dates coefficient of variation.

Phenolic compounds are directly linked to the peroxidase route expression, which participate in several physiological processes of great importance to the plant. Its rapid accumulation, oxidation and polymerization at the site of infection may limit the development of the pathogen. In this process, the phenols are oxidized by the action of hydrogen peroxide and polymerized to form lignin. This lignin is deposited on the cell wall and its reinforcement increases plant resistance to degrade enzymes/toxins produced by pathogens and acts as a physical barrier, reducing the severity of disease symptoms (Strack, 1997).

Phenols may occur in plant cells in their free form, in the form attached to the cell wall or conjugated to the formation of esters, amides or glycosides (Strack, 1997). Free phenols are easily extracted by the use of methanol and bounded by an esterification reaction with NaOH (Sodium hydroxide) (Kofalvi & Nassuth, 1995). However, glycosidic loop binding is stable to the bases, requiring acid hydrolysis or treatment with glucosidases to release the phenolic moiety (Hrazdina et al., 1997).

In this study, only free phenols were evaluated. Thus, the reduction in phenol expression may also have occurred due to the changes that led to the rapid increase in the concentration of both, free and bound cell wall phenols, which were not quantified. There may have been occurred rapid polymerization for the formation of lignin

bound to the cell wall, which led to the insolubilization of free phenols and a reduction in laboratory quantification in the subsequent hours (Labanca, 2002). For a consistent confirmation on this hypothesis, it would be essential to determine the concentration of conjugated phenols and lignin in the cell wall of the plant. A microscope evaluation would clarify this doubt, and eventually confirm the incorporation of phenols into the cell wall.

Plant responses to the pathogens are characterized by early accumulation of phenolic compounds into the site of infection (hypersensitivity reaction). The simple contact on the leaf surface of the pathogen is capable to stimulate the plant to exhibit defense responses. The hypersensitivity reaction role in interactions with biotrophic pathogens, such as powdery mildew, is great, since these pathogens form intimate associations of haustoria with host cells, causing cell death at the site of infection, can prevent them to have access to nutrients, which would lead to death (Hammerschimidt, 1992).

In a study carried out by Tarsis (2016), there was also an increase in the total phenolic compounds content, after the treatment with polysaccharides of residual basidiocarps cultivation from *L. edodes* (1.5 mg/mL) and inoculation with *X. gardneri*.

3.5 Peroxidases (POD)

In the biochemical evaluations, changes in peroxidase activity were observed. The results of Table 5 confirm what was shown in the previous results, and the activity of peroxidases was stimulated at 48 hours after application and 24 hours after inoculation with the pathogen for all treatments with MGF, similar to the data of total phenolic compounds.

The expression of this enzyme was increasing and peaked at 192 hours after application, for all the treatments with MGF. Biochemical analyzes of soybean leaves have demonstrated that peroxidases are good markers of resistance to the pathosystem in question, since there were differences in the accumulation of these enzymes between plants treated with MGF and plants treated with only water.

The growth in the POD activity was not accompanied by an increase in phenol concentration as was initially expected (since phenols serve as a substrate for this enzyme). However, other studies have verified that the increase of peroxidases activity does not necessarily imply an increase in the concentration of phenols.

According to Soylu et al. (2003), during the incompatible interactions between plant-microorganisms or treatments with elicitors, increases in peroxidase activity are often associated with the progressive incorporation of phenolic compounds into the cell wall. Plant cell wall reinforcement increases plant resistance to degrade enzymes/toxins produced by pathogens and acts as a physical barrier, reducing the severity of symptoms.

Table 5. Activity of peroxidases (enzymatic unit.minute^{-1}) of soybean plants treated with MGF of *G. lucidum* and copper oxychloride

Time (hours)	Treatments				
	Control	Gano	GanoSA	GanoLig	Copper oxychloride
0	15.89 aB	17.33 aB	17.49 aC	16.33 aC	17.08 aBC
48	17.93 bB	29.92 aA	26.55 aB	24.10 aB	12.57 bC
96	15.76 cB	31.74 aA	28.72abAB	29.43abAB	24.131bA
192	23.82 bA	31.54 aA	33.54 aA	32.30 aA	22.46 bAB
**CV = 12.61%; ***CV = 14.74 %					

Note. Averages followed by the same lowercase horizontal letter, or upper case vertical, are not significantly different by Tukey's test, at the ($p \leq 0.05$) error probability level. The elicitors *G. lucidum* (Gano), *G. lucidum* + Salicylic Acid (GanoSA), *G. lucidum* + lignin (GanoLig), Copper oxychloride (1 L ha^{-1}), distilled water (control). * Treatments coefficient of selection. ** Collection dates coefficient of variation.

Changes to peroxidases activity are correlated with the resistance response in different pathological systems. Peroxidases are responsible for the removal of hydrogen atoms from hydroxycinnamic alcohols, whose radicals polymerize to form lignin (Cavalcanti et al., 2005).

Several processes related to plant defense have contribution from peroxidases activity, including reactions of hypersensitivity, lignification, suberization and phytoalexin production. The cell wall reinforcement with lignin and phenols increases the resistance to enzymatic degradation caused by pathogens and acts as a mechanical barrier to toxins entering and to penetration through protoplast (Pascholati, 2011).

The induction of POD activity has been verified in other pathosystems when testing natural compounds for disease control. When using extracts of the *Lentinula edodes* mushroom in cucumber to control *Colletotrichum lagenarium*, Di Piero and Pascholati (2003) verified an elevation in the synthesis of peroxidases and chitinases. Silva et al. (2007) also observed that the activity of peroxidases was increased by the application of aqueous *L. edodes* extract to tomato and also reducing bacterial wilt. In addition, the presence of *A. blazei* (= *A. subrufescens*) and reduction of the presence of wilted leaves by *Ralstonia solanacearum* was observed in eggplant plants (Silva et al., 2008). Garcia (2018) observed that suspension obtained from the *A. brasiliensis* (= *A. subrufescens*) mycelium exhibited a fungitoxic effect on *Plasmopara viticola*, also induced an increase in peroxidase activity at 48, 72 and 96 hours after treatment with grapes. There was also a reduction of the AUDPC of the downy mildew of grape.

3.6 Chitinase

The application of *G. lucidum* culture filtrate submitted to lignin elicitation caused a significant increase of chitinase at 48 and 96 hours after the application of MGF, however, a reduction of the values after these periods has occurred, as showed in the Table 5.

In this study, the data for β-1.3 glucanase enzyme activity did not present statistical significance. This may mean that the defense route through the activation of glucanases is not a preferred route in the soybean/powdery mildew. Many studies demonstrate the activation of the enzyme chitinase or glucanase, being common the preference for one of these enzymes for immediate expression by the plant. However, synergistic effects are also demonstrated.

The data presented match with results obtained for PAL enzymes, peroxidases and phenolic compounds. There was a greater accumulation of chitinase from 192 hours after application with Ganoderma + SA filtrate. The results suggest the elicitation of *G. lucidum* in liquid medium with SA stimulates the fungus to produce bioactive compounds, which when applied to the plants, are capable of inducing resistance.

Several studies with SA treatment of plants confirm their function as a signaling molecule during plant defense responses to abiotic and biotic stresses, and their function in increasing the production of secondary metabolites in plants. However, the interaction of SA with microorganisms is still not clearly understood. In *G. lucidum*, it has already been reported that treatment with SA can increase the accumulation of ganodermic acid, which is a potent secondary metabolite. Overall, the results indicate that SA can induce biosynthesis of secondary metabolites in fungi (Cao, 2017). These secondary metabolites produced by *G. lucidum* have potential to reduce plant diseases.

Table 6. Activity of chitinase (UAbs.min^{-1}mg protein^{-1}) from soybean plants treated with MGF of *G. lucidum* and copper oxychloride.

Time (hours)	Treatments				
	Control	Gano	Gano AS	GanoLig	Copper oxychloride
0	0.033 aA	0.024 aB	0.044 aB	0.048 aC	0.042 aB
48	0.066 abA	0.080 abB	0.045 bB	0.127 aB	0.054 abB
96	0.076 bA	0.085 bB	0.294 aA	0.228 aA	0.131 bA
192	0.041 cA	0.163 bA	0.280 aA	0.161 bAB	0.081 cAB
**CV = 52.34%; ***CV = 37.73%					

Note. Averages followed by the same lowercase horizontal letter, or upper case vertical, are not significantly different by Tukey's test, at the ($p \leq 0.05$) error probability level. The elicitors *G. lucidum* (Gano), *G. lucidum* + Salicylic Acid (GanoSA), *G. lucidum* + lignin (GanoLig), Copper oxychloride (1 L ha^{-1}), distilled water (control). * Treatments coefficient of selection. ** Collection dates coefficient of variation.

Chitinase and β-1.3 glucanase are hydrolytic enzymes which, by and large, act in synergism. Hydrolytic enzymes (chitinase, glucanase, protease and cellulase) are responsible for the lysis of phytopathogens through hyperparasitism. The antagonistic properties of hydrolytic enzymes against various phytopathogens play an important role in biocontrol. These enzymes are capable of breaking down glycosidic bonds in chitin. Therefore, they play a vital role in the control of many plant diseases, degrading the cell walls of phytopathogens (Kim et al., 2003, Van Loon et al., 1994; Van Loon & Van Strien, 1999).

The increase in the activity of the chitinase enzyme for the treatment with the *G. lucidum* filtrate submitted to lignin elicitation may be arise from recognition of the plant via molecular patterns associated with injuries (DAMPs). The plant may have recognized lignin, used in elution to *G. lucidum* in liquid medium, as a component that has been degraded from its own cell wall and thus activating a signal of pathogen infection.

The most recent discovered category of elicitors are the molecular patterns associated with injuries (DAMPs), endogenous lesion indicators. Molecules that play a role in the 'normal' metabolism of the plant indicate injuries when they, or their fragments, appear outside the cell due to tissue rupture and then may act as activators of the plant's immune system. Studies have shown satisfactory results with extracts prepared from algae, leaves or other parts of the plants, as well as protein lysates (Choi & Klessig, 2016; Gust et al., 2017).

Some studies have shown the activation of chitinases and glunacanases with the use of alternative compounds as elicitors. Di Piero et al. (2004) reported that the application of the aqueous extract of *L. edodes* basidocarp to cucumber caused a local and systemic accumulation of peroxidases and chitinases, but not glucanases. The authors also observed that after inoculation with *C. lagenarium*, pretreated plants tended to have a decrease in the activity of chitinases compared to those that were not inoculated. Fiori-Suzuki et al. (2008) verified an increase in the activity of this enzyme in passion fruit inoculated with *Xanthomonas campestris pv. passiflora* and treated with extracts of *L. edodes* and *A. blazei*.

3.7 Evaluation of Disease in Greenhouse

The mycelium growth filtrate improved the resistance of soybean plants against *E. diffusa* infection. In the greenhouse experiment, a significant difference was observed between treatments for mildew severity (Figure 1). The Area Under the Disease Progression Curve (AUDPC) is a criterion that represents the evolution of the disease, considering all the evaluations together and summarizing them in a single value. There was a protection of the soybean plants submitted to treatments with MGF. The elicitors reduced the infection, reducing the severity of the disease.

The lower severity (%) reached by the treatments may be observed when the AUDPC is evaluated and compared to the control. The lowest rates were obtained by the treatment with filtrate submitted to elicitation with SA and lignin, followed by the application of the filtrate without elicitation and the fungicide copper oxychloride. Such results allow to affirm that the MGF were as efficient as the fungicide, and did not differ significantly from filtration without elicitation.

The reduction in the severity of disease may be related to the recognition of the molecules of microbial origin and previous activation of plant defense mechanisms, through the induced resistance, or by a direct action of the compounds on the fungus.

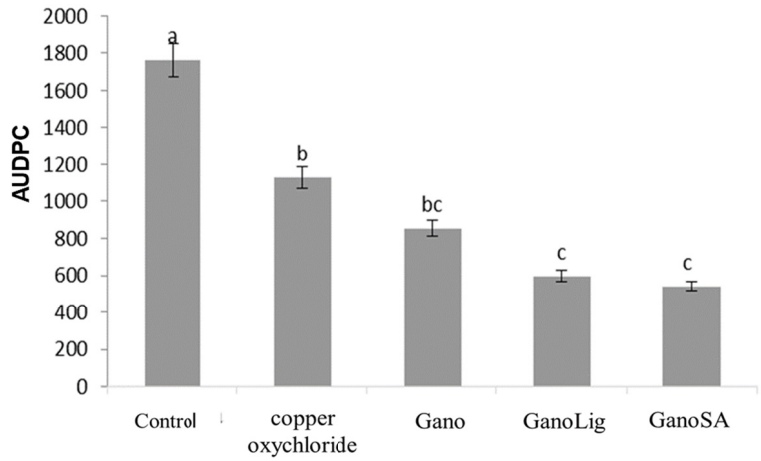

Figure 1. Area under the disease progress curve (AUDPC) caused by soybean powdery mildew (*Erysiphe diffusa*). Plants submitted to the application of elicitors, where different letters indicate statistical variation between treatments by the Tukey test, significant at 5% probability. The elicitors *G. lucidum* (Gano), *G. lucidum* + Salicylic Acid (GanoSA), *G. lucidum* + lignin (GanoLig), Copper oxychloride (1 L ha^{-1}) and distilled water (control)

The results obtained through the analysis of severity conform to the observed behavior through the enzymatic analysis. The application of MGF of *G. lucidum* provokes a viable defense response against powdery mildew in soybean plants, this behavior may be associated to the increase in the activities of PAL-enzyme, peroxidases, total phenols and chitinases, which could be acting on the plant-or even to the direct effect on inhibition of fungus development. The results obtained indicate the compounds of *G. lucidum* are efficient in reducing the severity of powdery mildew in soybean.

The antifungal properties of phenolic compounds also should be considered as a possible mechanism of inhibition of the powdery mildew development. As a potentially toxic phenylpropanoids, such as ferulic acid and coumaric acid, can be quickly formed and accumulate at the site of infection (Hammerschmidt, 1992).

Very few studies report the great potential of *Ganoderma* compounds in the control of plant diseases. A compound called G_app7 that was isolated from *Ganoderma applanatum* showed anti-oomycotic activity similar to strobilurin. This compound was effective in inhibiting the growth and development of mildew caused by *Sclerospora graminicola* in millet (*Pennisetum glaucum* (L.) R. Br.). Seed treatment with G_app7 resulted in a significant increase in protection against diseases (63%) under greenhouse conditions, compared to the control, which was water (Jogaiah et al., 2016). The potential of the basidiocarpos extracts of *Ganoderma sp.* and *Oudemansiella canarii* on the induction of systemic resistance in cucumber plants against powdery mildew (*Podosphaera xanthii*) were also reported in the studies of Stadnik and Bettiol (2001, 2007).

The use of elicitors is described in the literature as responsible for reducing the severity of diseases in several pathosystems. De Arruda et al. (2012) demonstrated the extracts of mushrooms *Agaricus blazei* (= *A. subrufescens*), *Lentinula edodes* and *Pycnoporus sanguineus* at 20% (v/v) concentration also have showed an effect on the control of powdery mildew in greenhouse. In studies by Piccinin et al. (2010), there was 34.53% reduction of leaf spot in sorghum (*Exserohilum turcicum* (*Pass.*) and *Colletotrichum sublineolum Ces* (*Wils*)), when *L. edodes* basidiocarp extract was applied 48 hours before inoculation. Assi (2007) confirmed 70% reduction in the severity of *Colletotrichum lindemuthianium* in bean, with the application of P. sanguineus aqueous extract at a concentration of 20% (v/v), being not statistically different from treatment with fungicide (axozystrobin 0.6 g.p.c/L). Di Piero (2003) found 100% reduction in the incidence of *passion fruit woodiness virus* (PWV) in passion fruit plants pretreated with the aqueous extract of *Agaricus blazei* (= *A. subrufescens*). The author also reported that extracts of *Lentinula edodes* applied 5 days before the inoculation of passion fruit gave significant local protection against PWV.

Search for efficient and sustainable strategies on the combat against plant pathogens is an absolute necessity for agriculture. With increasing investment in organic farming, where no chemical pesticides are used to control disease, studies that demonstrate the potential and efficiency of natural products to reduce disease intensity are of major relevance to academic and the rural milieu.

The results of this study show that the extracts of mushrooms have potential to be used in the control of plant diseases, either by the possible direct antimicrobial activity or by the activation of resistance mechanisms.

4. Conclusion

The mycelial growth filtrates of *G. lucidum* have potential to be used in the control of powdery mildew in the soybean crop. The MGF demonstrated the potential of induction of resistance in soybean plants, by the activation of phytoalexins, activation of the enzyme chitinase, phenylalanine ammonia-lyase increase of phenolic compounds and peroxidases activity. These effects have specificity in relation to activation time and association with elicitors.

The MGF have fungicidal potential when applied in soybean plants infected with powdery mildew, reducing the severity of the disease.

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