Fungitoxicity of the Pyroligneous Extract in the Development *in vitro* of *Colletotrichum gloeosporioides*

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Abstract

Products derived from renewable sources have been studied for their use in agriculture enabling increments in the production of agricultural culture, such as in the case of the pyroligneous extract. The research aimed at evaluating the fungitoxic potential of the pyroligneous extract of teak (*Tectona grandis* L.) on mycelial growth, sporulation and spore germination of *Colletotrichum gloeosporioides*. The experiment was conducted in completely randomized design with five treatments and four replications. Five doses of pyroligneous extract were tested (0, 25, 50, 100, 150 mL L^{-1}) following the *pour-plate* methodology in 10 mL of potato dextrose agar media and kept in the incubating room maintained at 25 ± 2 °C in a 12 hour-photoperiod for eight days. Mycelial growth variables (cm), mycelial index growth speed, growth inhibition (%), sporulation inhibition (%) and spore germination (%) were evaluated. There was a reduction for the mycelial growth as well as for the rate of mycelial growth, sporulation inhibition and spore germination as the doses were increased. The percentage inhibition of mycelial growth was 56 % for the highest dose (150 mL L^{-1}). Pyroligneous extract of teak has a direct fungitoxic action on growth, sporulation and germination *in vitro* of the fungus *C. gloeosporioides*.

Keywords: alternative control, anthracnose, spore germination, sporulation

1. Introduction

In modern agriculture, the viability of using products from renewable sources has been studied, enabling increases in the production of agricultural crops (Grewal et al., 2018).

One of these products is pyroligneous extract, obtained by condensation of the smoke released in the process of carbonization of wood for charcoal production. It is a yellow to reddish brown liquid and may be of different plant species (Porto et al., 2007), which gives it peculiar characteristics. This extract has been reported as an important antimicrobial, anti-inflammatory and antioxidant agent (Ibrahim et al., 2014). In addition, it is a source of chemical substances for the preservation, smoking and antimicrobial protection of various foods, mainly those of animal origin (Lee et al., 2010). This capacity for antimicrobial protection in food also gives it an excellent antifungal action against several phytopathogenic fungi (Oramahi et al., 2013; Macedo et al., 2019).

According to Loo et al. (2008) and Mmojieje and Hornung (2015), pyroligneous extract is generally composed of pyroligneous acid, which can be defined as an aqueous solution of acetic and formic acids, methanol and soluble tar, as well as other constituents. such as phenolic compounds, acids, neutral components, alcohols and micronutrients, most of which are water (85%). It has about 200 types of compounds, which interact synergistically promoting beneficial effect on plants, such as fungitoxicity to microorganisms that will attack the plant. When applied to soil, it improves physical, chemical and biological properties, favoring the absorption of nutrients by plants (Souza et al., 2012).

Research carried out in other countries and observations made in practice show that pyroligneous extract repels certain types of pests and prevents some diseases, including allowing the reduction of pesticide dosage (Kumar et al., 2011; Chauhan and Kang, 2013). However, there is still little scientific information about its effectiveness in soil, plants, pest control and the ideal concentrations to be used (Campos, 2007; Porto et al., 2007; Zhai et al., 2015). Rodrigues et al. (2020) studied the reduction in the development of *Colletotrichum gloeosporioides* due to the application of high dilutions of pyroligneous extract, in a homeopathy study, verifying potentialities.

Pyroligneous extract has attracted the attention of researchers and technicians from various fields, mainly agronomic, as an alternative to a more natural product when compared to organosynthetic compounds. It should be noted that the use of pyroligneous extract in agriculture, although its proven effect on plant pest and disease control (Macedo et al., 2019; Rodrigues et al., 2020), still needs further information and further studies (Campos, 2007).

According to Saigusa (2002), the activating or inhibiting effect of pyroligneous extract on living organisms depends on its concentration. The author reports that, in the case of microorganisms, the solution has an immediate and short-lasting effect, but is effective in restoring vitality and at the same time strengthening the defense system that exists in the plant, thus reducing the level of damage caused by microorganisms. and can be adopted in conjunction with other agroecological practices. This behavior is due to the composition of the pyroligneous extract, which contains a large amount of chemical compounds, which can

have positive effects on the plant's ability to resist attack by insects and also diseases (Souza et al., 2018).

One of the species widely exploited in silviagricultural activities that could be used to produce pyroligneous extract is teak (*Tectona grandis*). During its use and processing, a large volume of wood waste is generated, which can be destined for charcoal production, and consequently in the conversion of smoke to pyroligneous extract. Teak chemical studies report the presence of a substance belonging to the tectoquinone class, present in adulthood, which is attributed some antifungal, bactericidal and insect repellent properties of some insects, conferring high durability when exposed to the rigors of time (Ragunathan et al., 1996).

In this context, the present research aimed to evaluate the fungitoxicity of doses of teak pyroligneous extract (*Tectona grandis*) on mycelial growth, sporulation and in vitro spore germination of *Colletotrichum gloeosporioides*.

2. Material and Methods

The experiment was conducted at the Microbiology Laboratory of Mato Grosso State University, Campus Alta Floresta, MT, Brazil, in the year 2014. The research comprised two stages, the first to verify the in vitro fungitoxic action of pyroligneous extract doses on mycelial growth and sporulation of the fungus *Colletotrichum gloeosporioides* and the second to verify these doses on spore germination.

Obtaining pyroligneous extract

The pyroligneous extract was obtained from teakwood residue (*Tectona grandis*) at the Wood Technology Laboratory of the Mato Grosso State University, Campus Alta Floresta, through the carbonization process in a brick oven at 180 °C.

The pyroligneous *extract* obtained was stored in amber glass for a little over four years, enough time for the extract to decant and separate into phases, and the upper phase contains light oils, the central phase the pure pyroligneous and the lower phase the tar (Chauhan and Kang 2013), dispensing with purification processes to eliminate possible compounds that could compromise its use, such as high tar content.

With the separation of the extract in phases by the decantation process, the central part of the decanted liquid, comprising the pyroligneous portion, with pH close to 3.05 was used in the experiment.

Obtaining the pathogen Colletotrichum gloeosporioides

Leaves of cashew trees (*Anacardium occidentalle*) present in the study region, with symptoms of anthracnose disease, were collected and taken to the Microbiology Laboratory to isolate the causal agent. Isolation was performed by adopting standard procedures for isolation in potato-dextrose-agar (BDA) culture medium, according to the method described by Alfenas and Mafia (2007).

After this procedure, the fungus was subcultured to other Petri dishes until pure culture was

obtained, keeping them in a BOD germination chamber, with a photoperiod of 12 hours at 25 °C.

In vitro biotests for antifungal activity determination

The first stage involved the mycelial growth and sporulation tests, arranged in a completely randomized design (DIC) with five treatments (doses of pyroligneous extract) and four replications each, and the repetition consisted of seven sample units (Petri dishes).

The second stage involved the spore germination inhibition test, organized in a completely randomized design (DIC) with five treatments (doses of pyroligneous extract) and four replications each, the repetition represented by a microscopy slide.

Mycelial Growth Inhibition Test

To verify fungitoxicity in mycelial growth and sporulation, pyroligneous teak extract (EPt) was used in five dosages (0, 25, 50, 100 and 150 mL L^{-1}), following the pour-plate methodology, in which the The proportional amount of that dose was deposited in 9 cm diameter Petri dishes and then 10 mL of the flux culture medium (BDA) was poured, thus completely homogenizing the medium.

After medium solidification, a 10 mm disc of pure culture, previously incubated for 10 days in BDA medium, was deposited in the center of the plate. These plates were then sealed with plastic film and kept in an incubation room at 25 ± 2 °C, with a 12 hour photoperiod.

The evaluations were performed daily after 48 hours of inoculation, by measuring the diameter of the colonies (average of two diametrically opposite measurements) with the aid of a millimeter ruler, obtaining an average for each repetition, until the time of fifteen plaques. Petri (50% + 1 of the total plates) from the same treatment presented minimal colonization of ¾ of the middle surface.

The response variables obtained in the mycelial growth inhibition test were:

• Fungal growth: determined from mycelial growth values, using the means of the last observation of each repetition of the respective treatment.

• Mycelial growth velocity index (IVCM): obtained from the averages of daily mycelial growth values of each treatment (Equation 1), as described by Souza et al (2007):

$$IVCM = \Sigma \frac{(D - Da)}{N} \qquad \text{Equation 1}$$

were:

D = current average diameter of the colony;

Da = average diameter of the previous day colony;

N = number of days after inoculation

• Mycelial Growth Inhibition Percentage (PIC): obtained through the final repetition growth averages, which compares the mean diameter (cm) of the treatments relative to the control, using the Equation 2, described by Nascimento et al. (2013):

$$PIC = \left[\frac{(\text{Ø da testemunha} - \text{Ø do tratamento})}{\text{Ø da testemunha}}\right] x\ 100$$

Equation 2

Sporulation Inhibition Test

The sporulation inhibition test was performed at the end of the mycelial growth inhibition test. For the determination of spore concentration per milliliter, five random plate spore solution was used from each treatment, in which 10 mL of sterile distilled water was added per plate. With the aid of the Drigalski's loop, the friction was performed on the mycelium, followed by gauze filtration.

Then, a 100 µL aliquot of the pure solution from the five plates was deposited in a Neubauer chamber and observed under an optical microscope for spore counting.

After spore counting, data were entered into the computer program Calibra® version 2011, available from Embrapa Environment, to determine spore concentration per milliliter (Santos et al. 2011).

Germination Test

In order to evaluate the period required for the maximum germination of *C. gloeosporioides* spores, a spore germination curve was established, relating the germination percentage to the time required for germination.

For this test, a 100 µL aliquot of the spore suspension (1.7×10^6 spores mL^{-1}) deposited in a Neubauer chamber was used and incubated in a BOD wet chamber in the absence of light at 25 °C for 2, 4, 6, 8 and 10 hours, totaling five evaluation periods, with four repetitions each. Observations were made under an optical microscope at the end of each period, counting the germinated spores.

To determine the inhibition of spore germination, 100 µL aliquots, consisting of 50 µL of the spore suspension (8.5×10^5 spores mL^{-1}) plus 50 µL of each pyroligneous extract dose (0, 25, 50, 100 and 150 mL L^{-1}) were placed on a microscopy slide coated with a thin 1% agar-water layer. The four slides of each treatment were incubated in a humid chamber in the dark, kept in BOD at 25 ºC for eight hours.

The criterion for considering germinated spore was as described by Ulloa and Hanlin (2000), who describes the germ tube as short hyphae that grows from the germ pore during germination and which develops continuously under favorable conditions, forming a hyphae. Longer length (mycelium), being also considered as a new assimilative phase of the fungus. From the point of view of this experiment, the complete germ tube was considered when its length was equal to or greater than the spore width.

Then, next to the optical microscope, the test was evaluated by photographic recording with

digital eyepiece and objective with 400x magnification. Subsequently, a grid (2 x 2 cm) was projected on these images in order to randomize the count of germinated spores, being counted 100 spores for each repetition.

The values obtained for mycelial growth, mycelial growth rate index, growth inhibition percentage, inhibition and spore germination curve were subjected to analysis of variance by the F test, and regression analysis was performed for the concentrations tested with a significance level of 1% through the statistical program Sisvar® (Ferreira 2014). For the inhibition of sporulation, a descriptive comparison of the observed means was performed.

3. Results and Discussion

Gradual reduction in mycelial growth was observed as the concentration of teak pyroligneous extract increased (Figure 1).
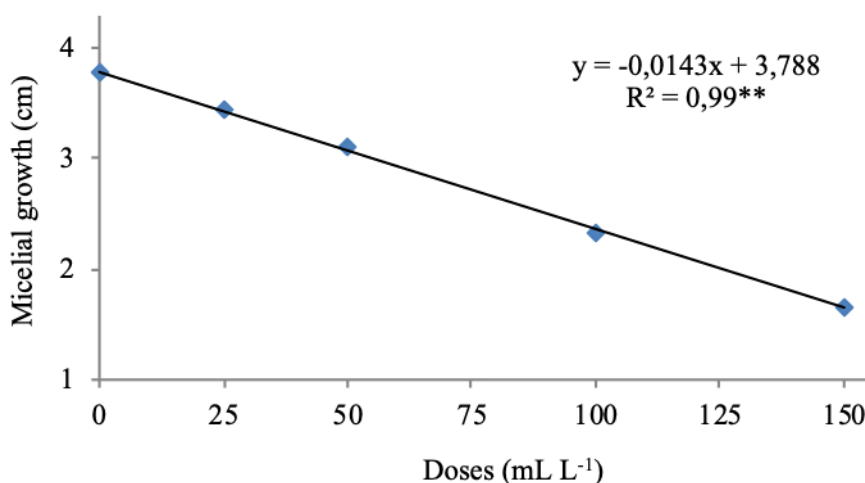

Figure 1. Mycelial growth (cm) of the fungus *Colletotrichum gloeosporioides* under doses of teak pyroligneous extract (*Tectona grandis*) after eight days of incubation. CV (%): 2.87

Kumar et al. (2011) found similar behavior in the mycelial growth of the fungus *Coleosporium* sp. when submitted to the doses of pyroligneous oak extract, observing reduction of myceliation according to the increase of the dosage. The best results were observed in the 50% solution concentration, where 32% of spores were inhibited. This behavior was also observed in this study, with high mycelial growth for the control (3.78 cm), and a reduction of this growth as the dosage of the extract with lower myceliation at the highest dose (1.64 cm) was increased after eight days. inoculation.

Also similarly, Theapparat et al. (2015), testing pyroligneous wood extract (*Eucalyptus camaldulensis*, *Leucaena leucocephala*, *Azadirachta indica*), rubber tree (*Hevea brasiliensis*), and bamboo (*Dendrocalamus asper*) and their antifungal capacity against two white rot fungi (*Trametes versicolor* and *Rigidoporopsis rylidoporopsis*) brown rot fungus (*Gloeophyllum trabeum*) and a frogeye spot fungus (*Botryodiplodia theobromae*) found that pyroligneous acids from both species exhibited antifungal activity (growth inhibition, minimal inhibitory concentration, and minimal fungicidal concentration), especially bamboo and rubber, which

presented higher total phenolic concentrations. This demonstrates the potential of pyroligneous extract in antifungal action, with diverse ability to prevent the development of these microorganisms.

In this study, when comparing the research by Theapparat et al. (2015), it appears that, within the values observed in the same dose (100 mL L^{-1}), there was mycelia (2.36 cm) (Figure 1), but a value lower than the previous dose. In this way, it is also possible to lower the potential for fungal control of this pyroligneous extract under these conditions.

The mycelial growth velocity index (IVCM) can be observed in Figure 2, verifying that the growth rate of the fungus in the control (index 0.55) is almost twice higher than in the highest dose (index 0.28). Rodrigues et al. (2020) found for the same fungus, a reduction in the speed of sporulation of the fungus of around 49%.

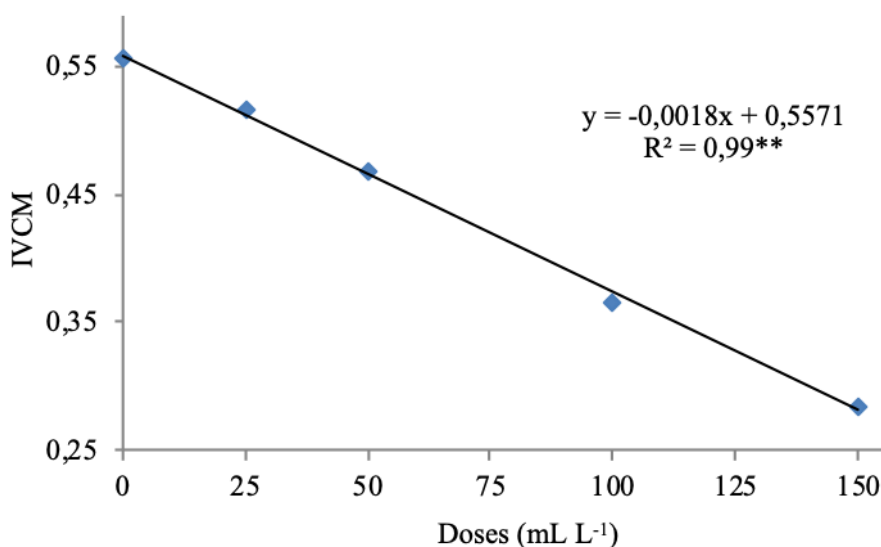
$$y = -0,0018x + 0,5571$$
$$R^2 = 0,99**$$

Figure 2. Mycelial growth rate index (IVCM) of the fungus *Colletotrichum gloeosporioides* under doses of teak pyroligneous extract (*Tectona grandis*) with eight days of incubation. CV (%): 3.16

Increasing doses of pyroligneous extract promoted a higher percentage of mycelial growth inhibition (PIC), with a maximum inhibition rate of 56.31% for the highest dose (150 mL L^{-1}) when compared to the control (Figure 3).
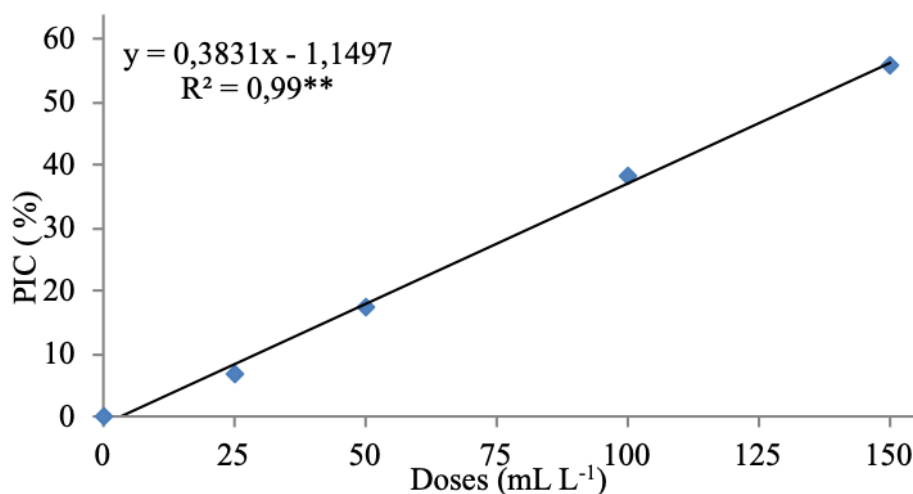
Figure 3. Percentage of mycelial growth inhibition (PIC) of the *Colletotrichum gloeosporioides* fungus under doses of teak pyroligneous extract (*Tectona grandis*) after eight days of incubation. CV (%): 9.56

The three characteristics analyzed for the evaluation of mycelial growth inhibition of *C. gloeosporioides* as a function of the use of teak pyroligneous extract showed convergent behavior and demonstrated its efficiency in reducing fungal development, confirming literature information on the potential of the fungus product for use as a phytosanitary agent (Macedo et al., 2019; Rodrigues et al., 2020).

As in the mycelial growth inhibition test (Figure 1), there were also changes in sporulation rates (Figure 2) of *C. gloeosporioides* fungus, varying according to the applied dosage.

A maximum sporulation peak (11×10^5 spores mL^{-1}) is observed in Figure 4 when 50 mL L^{-1} is used. This fact probably occurs because when in smaller dosages the pyroligneous extract can promote a stress to the fungus, where the fungus, as a mechanism of attack and survival, increases spore production, which later ensures greater chances of colonization and dispersal.

This fact was evident in other experiments with alternative controls, as observed by Balbi-Peña et al. (2006a), verifying the efficiency of turmeric extracts in *Alternaria solani*, where the 1% extract concentration inhibited 10% more spore production than when compared to the 10% concentration. Moura et al. (2012), with different concentrations of lemongrass extract in the sporulation rate of *C. gloeosporioides* fungus, found that the concentration of 25% the number of spores doubled compared to the previous concentration of 20% (1.0×10^4 spores mL^{-1}).

The 50 mL L^{-1} dose promoted intermediate mycelial development (Figure 1) that is, there was restriction in the average mycelial growth when compared to the control; however it provided a 74% increase in spore production compared to the control.
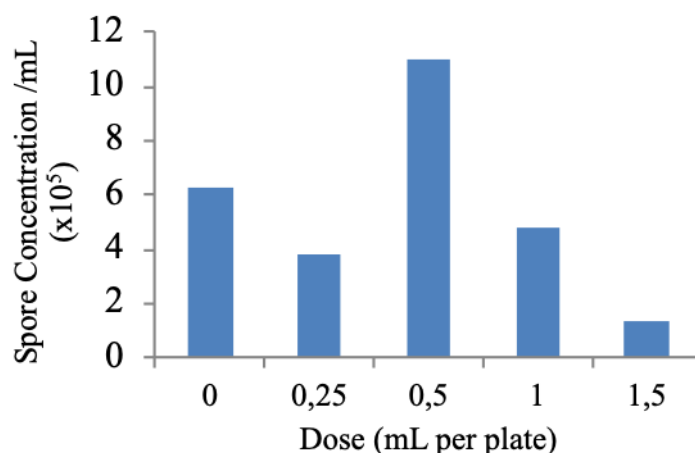
Figure 4. Sporulation of the fungus *Colletotrichum gloeosporioides* after mycelial growth in medium plus doses of teak pyroligneous extract (*Tectona grandis*) after eight days of incubation

The lowest spore concentration occurred at 150 mL L^{-1}, with 1.3 x 10^5 spores mL^{-1}; therefore the mycelial growth limitation (1.64 cm) (Figure 1) reached a fungistatic level, which reduced the sporulation in this treatment by about 80% when compared to the control (6.3 x 10^5 spores mL^{-1}).

The in vitro germination curve of *C. gloeosporioides* spores can be observed in Figure 5, which shows that the maximum germination occurred after 8.8 hours of incubation (69.87%), very close to the preceding periods, showing that from the six hours of incubation reaches levels above 60% germination, (6 hours 62% and 8 hours 67%).

Determining the appropriate incubation period for phytopathogenic fungal spore germination is of paramount importance, as it allows for optimal use of time in experiments and ensures success when studying the development and physiology of the local species under study. The literature shows divergence regarding this period, as observed in several studies such as Kumar and Kumar (1980), who report about six hours of incubation for *Alternaria alternata*, *Curvularia pallescens* and *Drechslera australiensis*; Celoto et al. (2008) and Moura et al. (2012) describe a nine hour period for *C. gloeosporioides*; Balbi-Peña (2006b) reports a twelve-hour period for *Alternaria solani*.

Generally high concentrations of *C. gloeosporioides* spores and other species of the genus, are attributed to the presence of accumulation of extracellular material, called mucilage (orange sputum with large amount of spores) commonly found in in vitro fungal colonies, or under conditions favorable environmental conditions, which is involved in the survival, dispersal and pathogenicity of species of this genus. In vitro, mucilage production can vary according to the incubations periods, isolation and culture conditions.

http://jas.macrothink.org

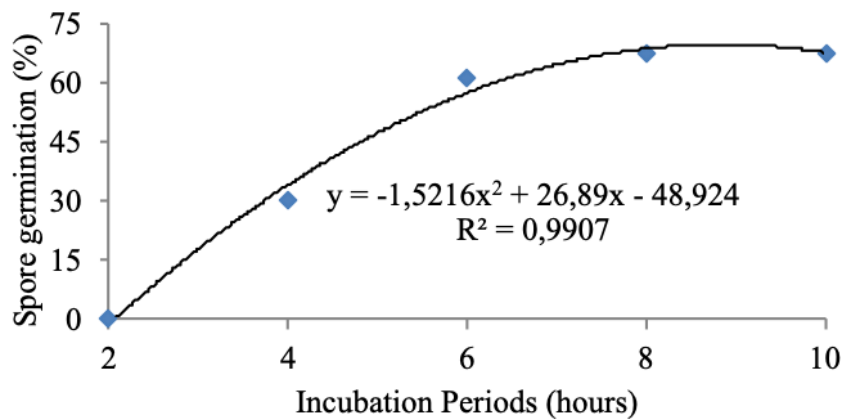$$y = -1{,}5216x^2 + 26{,}89x - 48{,}924$$
$$R^2 = 0{,}9907$$

Figure 5. *Colletotrichum gloeosporioides* spore germination curve over time. CV (%): 19.57

According to Ferreira et al. (2009), conidial mucilage would have the ability to control spore germination. Thus, there is a possibility that, when acerulate suspension or high concentration occurs, germination self-inhibition may occur due to the presence of the substance called mycosporin-alanine, which can be attributed to the fact of germination in the curve. However, it is worth noting that the mechanisms involved in inhibiting the action of microsporin-alanine are complex and depend on many variables, requiring further studies.

According to the results obtained, after eight hours of incubation, germination is greater than 60%, and this time interval is satisfactory to evaluate the effect of fungitoxic products on *C. gloeosporioides* spore germination.

Figure 6 shows the effect of pyroligneous extract doses on the spore germination process. Spore germination decreased with increasing dose, with a germination peak of 98% for zero dose and a minimum value of 86% when applied 150 mL L^{-1}. A reduction in germination rate close to 13% was found between the lowest and highest dose.

$$y = -0{,}0822x + 98{,}14$$
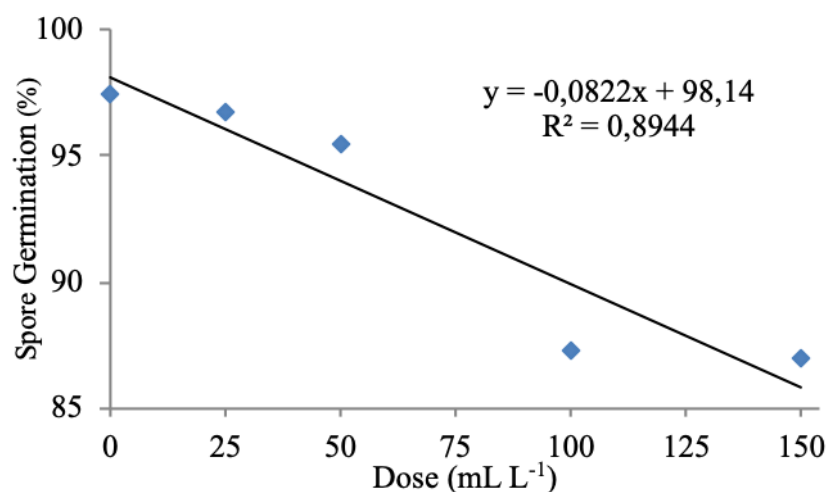$$R^2 = 0{,}8944$$

Figure 6. Germination of *Colletotrichum gloeosporioides* spores under doses of teak pyroligneous extract (*Tectona grandis*) after eight hours of incubation. CV (%): 3.79

It was noted that the 25 and 50 mL L^{-1} dosages presented low percentages of germination

inhibition (below 3%) when compared to the control. Low dosages that behave similarly or close to the control in the germination process might not interfere with the process of infection (fungal penetration into the plant), as reported by Balbi-Peña et al. (2006a), and would be acting through other mechanisms in later stages of the pathogen-host relationship cycle, such as colonization.

The results obtained in relation to the germination (Figure 6) and sporulation (Figure 4) of *C. gloeosporioides* corroborate the results found in the mycelial growth (Figure 1) evaluations of this study regarding the action of teak pyroligneous extract as a fungitoxic agent in vitro, being the first step to identify the potential of this product in the control of phytopathogens. However, in vivo research should be performed to prove its efficiency, evaluating its response when interacting with other factors in field cultivation situations.

5. Conclusion

Pyroligneous teak extract, under the conditions under which the research was conducted, presents fungistatic action on mycelial growth, sporulation and spore germination of *Colletotrichum gloeosporioides*.

References

Alfenas, C. A., & Mafia, R. G. (2007). *Métodos em fitopatologia*. Viçosa: UFV, 382p.

Balbi-Peña, M. I. et al. (2006a). Controle de *Alternaria solani* em tomateiro por extratos de *Curcuma longa* e curcumina – I. Avaliação *in vitro*. *Fitopatologia Brasileira*, *31*, 310-314. https://doi.org/10.1590/S0100-41582006000300012

Balbi-Peña, M. I. et al. (2006b). Controle de *Alternaria solani* em tomateiro por extratos de *Curcuma longa* e curcumina - II. Avaliação *in vivo*. *Fitopatologia Brasileira*, *31*, 401-404. https://doi.org/10.1590/S0100-41582006000400012

Campos, A. D. (2007). Técnicas para produção de extrato pirolenhoso para uso agrícola. *Circular Técnica 65*. Embrapa: Pelotas.

Celoto, M. I. B. et al. (2008). Atividade antifúngica de extratos de plantas a *Colletotrichum gloeosporioides*. *Acta Scientiarum Agronomy*, *30*, 1-5. https://doi.org/10.4025/actasciagron.v30i1.1104

Chauhan, A. K., & Kang, S. C. (2013). In vivo control of perilla rust disease by oak pyroligneous liquor. *Journal of Agricultural Chemistry and Environment*, *2*, 86-89. https://doi.org/10.4236/jacen.2013.24013

Ferreira, D. F. (2014). Sisvar: a Guide for its Bootstrap procedures in multiple comparisons. *Ciência e Agrotecnologia*, *38*,109-112. https://doi.org/10.1590/S1413-70542014000200001

Ferreira, J. B. et al. (2009). Eventos do processo de infecção de *Colletotrichum gloeosporioides* inoculados em folhas de *Coffea arabica* L. *Summa Phytopathologica*, *35*, 273-281. https://doi.org/10.1590/S0100-54052009000400004

Grewal, A. et al. (2018). Production, prospects and potential application of pyroligneous acid

in agriculture. *Journal of Analytical and Applied Pyrolysis*, *135*(1), 152-159. https://doi.org/10.1016/j.jaap.2018.09.008

Ibrahim, D. et al. (2014). Evaluation of antibacterial effects of *Rhizophora apiculata* pyroligneous acid on pathogenic bacteria. *Malaysian Journal of Microbiology*, *10*(3), 197-204. https://doi.org/10.21161/mjm.60714

Kumar, R., & Kumar, S. (1980). Effect for certain homoeopathic medicines on fungal growth and conidial germination. *Indian Phytopathology*, *33*,620-621.

Kumar, V. et al. (2011). Inhibitory activity of oak pyroligneous liquor against *Coleosporium plectranthi*, an obligate parasite responsible for the rust disease on perilla leaf. *Korean Journal of Environmental Agriculture*, *30*, 453-458. https://doi.org/10.5338/KJEA.2011.30.4.453

Lee, S. H. et al. (2010). Production of pyroligneous acid from lignocellulosic biomass and their effectiveness against biological attacks. *Journal of Applied Sciences*, *20*, 2440-2446. https://doi.org/10.3923/jas.2010.2440.2446

Loo, A. Y. et al. (2008). Antioxidant activity of compounds isolated from the pyroligneous acid, *Rhizophora apiculate*. *Food Chemistry*, *107*, 1151-1160. https://doi.org/10.1016/j.foodchem.2007.09.044

Macedo, D. G. C. et al. (2019). Study of the control of fungus occurring in *Schizolobium amazonicum* seeds with the use of pyroligneous extract. 2019. *International Journal of Plant & Soil Science*, *31*, 1-9. https://doi.org/10.9734/ijpss/2019/v31i430216

Mmojieje, J., & Hornung, A. (2015). The potential application of pyroligneous acid in the UK agricultural industry. *Journal of Crop Improvement*, *29*, 228-246. https://doi.org/10.1080/15427528.2014.995328

Moura, G. S. et al. (2012). Controle da antracnose em maracujá-amarelo por derivados de capim-limão (*Cymbopogon citratus*). *Arquivos do Instituto Biológico*, *79*, 371-379. https://doi.org/10.1590/S1808-16572012000300007

Nascimento, J. M. et al. (2013). Inibição do crescimento micelial de *Cercospora calendulae* Sacc. por extratos de plantas medicinais. *Revista Brasileira de Plantas Medicinais*, *15*, 751-756. https://doi.org/10.1590/S1516-05722013000500016

Oramahi, H. A., & Yoshimura, T. (2013). Antifungal and antitermitic activities of wood vinegar from *Vitex pubescens* Vahl. *Journal of Wood Science*, *59*, 344-350. https://doi.org/10.1007/s10086-013-1340-8

Porto, P. R. et al. (2007). Efeito da aplicação do extrato pirolenhoso na germinação e no desenvolvimento de mudas de *Pinus elliottii* var. *elliottii*. *Instituto Florestal*, *31*, 15-19.

Ragunathan, R. et al. (1996). Cultivation of *Pleurotus* spp. on various agro-residues. *Food Chemistry*, *55*, 39-44. https://doi.org/10.1016/0308-8146(95)00079-8

Rodrigues, C. et al. (2020). Evaluation of high dilutions extract of pyroligneous on the

development of *Colletotrichum gloeosporioides*. *Ibero-American Journal of Environmental Sciences*, *11*, 69-77. https://doi.org/10.6008/CBPC2179-6858.2020.002.0008

Saigusa, T. (2002). *Aplicação de extrato pirolenhoso na agricultura* (APAN – Associação dos produtores de Agricultura natural). Apostila, p.24-32.

Santos, E. R. et al. (2011). *Sistema para contagem de esporos microbianos e calibração de suspensão* (CALIBRA). Embrapa Meio Ambiente, Jaguariúna,.

Souza, B. O. et al. (2007). Determinação da variabilidade em isolados de *Colletotrichum lindemuthianum* por meio de marcadores morfológicos e culturais. *Ciência e Agrotecnologia*, *31*, 1000-1006. https://doi.org/10.1590/S1413-70542007000400009

Souza, J. B. G. et al. (2012). Characterization of pyroligneous acid used in agriculture by gas chromatography-mass spectrometry. *Journal of the Brazilian Chemical Society*, *23*, 610-617. https://doi.org/10.1590/S0103-50532012000400005

Souza, J. L. S. et al. (2018). Potential antimicrobial of pyroligneous extracts – A systematic review and technological prospecting. *Brazilian Journal of Microbiology*, *49S*, 128-139. https://doi.org/10.1016/j.bjm.2018.07.001

Theapparat, Y. et al. (2015). Pyroligneous acids from carbonisation of wood and bamboo: their components and antifungal activity. *Journal of Tropical Forest Science*, *27*, 517-526.

Ulloa, M., & Hamlin, R. T. (2000). *Illustrated Dictionary of Mycology*. American Phytopathological Society Press, Minnesota, 448p.

Yataga, M. (2004). Biological activity of wood vinegar and its recent applications. *National Food Industry*, *46*, 5-16.

Zhai, M. et al. (2015). Chemical compositions and biological activities of pyroligneous acids from walnut shell. *BioResources*, *10*, 1715-1729. https://doi.org/10.15376/biores.10.1.1715-1729