Special Supplement: *Cerrado* **[Brazilian Savanna]**

Efficiency of a new *Waitea circinata* extract against rice pathogens[1]

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ABSTRACT

Waitea circinata (Warcup & Talbot) is an orchid antagonist mycorrhizal fungus with biocontrol potential against rice pathogens. This study aimed to optimize the extraction method, obtain a new extract and evaluate its efficiency against rice pathogens *in vitro* and *in vivo*, as well as to compare it with other extraction methods and *W. circinata*. The extracts were obtained and screened for *in vitro* growth inhibition against the pathogens *Cochliobolus miyabeanus*, *Monographella albescens* and *Sarocladium oryzae*, using the following extracts: mycelial, crude, lyophilized and mycelial mass. An additional *in vitro* assay was performed with the principal rice pathogen (*Magnaporthe oryzae*), in order to evaluate the conidial germination and appressorium formation. Based on this evaluation, the lyophilized and mycelial mass extracts were tested *in vivo* against rice blast (*M. oryzae*) and compared to the *W. circinata* mycelial suspension, in different application forms (simultaneous and previous). The mycelial mass extract inhibited all the pathogens, and the crude and lyophilized extracts inhibited *C. miyabeanus* and *M. albescens*, respectively. The mycelial mass extract inhibited the *M. oryzae* conidial germination and appressorium formation by 80 %, and the simultaneous and previous applications suppressed the rice blast by 94 %. These results indicate that the new extract can be used to control rice pathogens.

KEYWORDS: Biocontrol, leaf scald, brown spot, sheath rot, rice blast.

RESUMO

Eficiência de um novo extrato de *Waitea circinata* contra patógenos de arroz

Waitea circinata (Warcup & Talbot) é um fungo antagonista micorrízico de orquídea, com potencial de biocontrole contra patógenos de arroz. Objetivou-se otimizar o método de extração, obter um novo extrato e avaliar sua eficiência contra patógenos de arroz *in vitro* e *in vivo*, bem como compará-lo com outros métodos de extração e *W. circinata*. Os extratos foram obtidos e testados para inibição de crescimento *in vitro* contra os patógenos *Cochliobolus miyabeanus*, *Monographella albescens* e *Sarocladium oryzae*, utilizando-se os seguintes extratos: micelial, bruto, liofilizado e de massa micelial. Um ensaio adicional *in vitro* foi realizado com o principal patógeno do arroz (*Magnaporthe oryzae*), para avaliar a germinação de conídios e a formação de apressórios. Com base nessa avaliação, os extratos liofilizado e de massa micelial foram testados *in vivo* contra a brusone do arroz (*M. oryzae*) e comparados com a suspensão micelial de *W. circinata*, em diferentes formas de aplicação (simultânea e prévia). O extrato de massa micelial inibiu todos os patógenos, e os extratos bruto e liofilizado inibiram *C. miyabeanus* e *M. albescens*, respectivamente. O extrato de massa micelial inibiu a germinação de conídios e a formação de apressórios de *M. oryzae* em 80 %, e as aplicações simultâneas e prévias suprimiram a brusone em 94 %. Os resultados indicam que o novo extrato pode ser usado para controlar patógenos de arroz.

PALAVRAS-CHAVE: Biocontrole, escaldadura, mancha parda, podridão da bainha, brusone.

INTRODUCTION

Brown spot (*Cochliobolus miyabeanus*), leaf scald (*Monographella albescens*), sheath rot (*Sarocladium oryzae*) and rice blast (*Magnaporthe oryzae*) affect the yield in upland and irrigated rice (Fisher et al. 2012, Laha et al. 2017). The recorded losses in grain yield are 100 % (rice blast), 90 % (brown spot), 30 % (leaf scald) and 90 % (sheath rot) (Ou 1985, Rao 1996, Prabhu et al. 2009, Sunder et al. 2014). Although the disease management integrates genetic resistance, cultural practices and chemical

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control, fungicides are widely applied. In Brazil, the pesticide consumption reached 549,280 tons in 2018 (Ibama 2018), but the excessive use of fungicides causes environmental pollution, resistance of the pathogen to molecules, decrease in the number of non-target organisms, poisoning and cancer (Bozdogan 2014, Nascimento et al. 2020), being very important the search for disease control alternatives that are sustainable.

A great effort has been made to use bioagents in the field that are safe for consumers and for the environment, as well as more efficient than the existing ones and independent of the variability of pathogens (Oliveira et al. 2019, Chaibub et al. 2020). However, the efficacy of biological control agents may be affected by preparations formed with spore suspensions, mycelial cultures with media, pellet or powder/granular and extract formulations (Narayanasamy 2013, Carvalho et al. 2015).

Extracts are a set of molecules obtained from plants or microorganisms, and fungal metabolites are widely known to exhibit a broad range of biological properties, including antimicrobial activity (Synytsya et al. 2017).

Mycorrhizal fungi interact with hosts forming spiral complexes of fungal hyphae, called pelotons, which form during the symbiotic interaction with many orchid species (Rasmussen & Rasmussen 2009, Sousa et al. 2019, Yeh et al. 2019). The orchid mycorrhizal fungi *Waitea circinata* is obtained from roots of the Brazilian Savanna orchid *Epidendrum nocturnum*. It is a non-obligate symbiont that acts as a biocontrol agent against plant pathogens (Mosquera-Espinosa et al. 2013, Carvalho et al. 2015), being an option for the formulation of bioproducts.

W. circinata inhibits *in vitro M. oryzae* and its crude extract suppresses *in vivo* rice blast (Carvalho et al. 2015). However, the process is not adequate, because it takes 26 days, and a large amount of solvent is required (Carvalho et al. 2015). Thus, it is necessary to explore a reasonable extraction method.

There is no registered or recommended biological product from orchid mycorrhizal fungi or extracts to control these diseases (Agrofit 2021). The advantages of obtaining a bioproduct in the extract or isolated metabolite is the lack of need for cooling and a probable increase in shelf life due to a greater stability (Carvalho et al. 2015). Therefore, this study aimed to optimize the extract method, obtain a new extract and evaluate its efficiency against rice pathogens *in vitro* and *in vivo*, as well as to compare it with other extraction methods and *W. circinata*.

MATERIAL AND METHODS

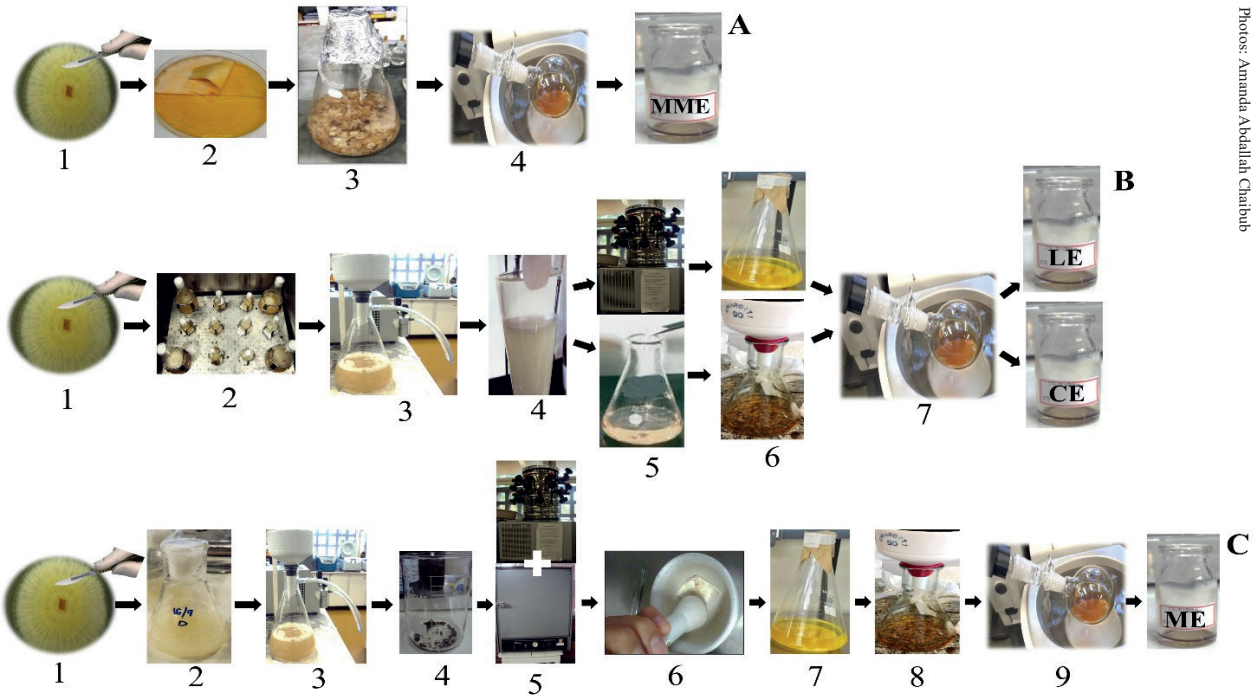
The *W. circinata* was obtained from rupicolous *E. nocturnum* orchid from the Brazilian Savanna (Sousa et al. 2019) and belongs to the Microorganisms Genetics Laboratory of the Universidade Federal de Goiás (Goiânia, Goiás state, Brazil). The rice pathogen isolates (*Cochliobolus miyabeanus* - BRM 45114, *Monographella albescens* - BRM 32184, *Sarocladium oryzae* - BRM 6461 and *Magnaporthe oryzae* - BRM 31295) belong to the Multifunctional Collection of Microorganisms of the Empresa Brasileira de Pesquisa Agropecuária (Embrapa Arroz e Feijão). The assays were performed from May 2014 to July 2016.

The methodology applied to obtain the mycelial, crude and lyophilized extracts has been previously described by Carvalho et al. (2015).

The mycelial mass extract was obtained from *W. circinata* cultivation in PDA for 11 days, under continuous light, at 26 ± 2 ºC. The mycelium was scraped with a scalpel to remove the excess of culture medium and 16 g of the mycelium were thawed, placed in flasks and macerated with 100 mL of ethyl acetate for cold extraction for 3 days. Filtration was then performed to remove the solid part using a Whatman nº 4 filter paper under vacuum, and the solvent was removed on a rotary evaporator under reduced pressure for 1 h. These procedures were repeated three times to obtain the mycelial mass extract (Figure 1).

The inhibition assays were performed using a completely randomized design, with six treatments and five replications for each pathogen. The microorganisms were grown in PDA for 11 days at 25 ºC. Five dilutions of each extract were prepared. First, the samples were weighed and diluted in 2 mL of ethanol. Next, Milli-Q™ water was added to obtain the final concentration. The samples were then filtered through a syringe fitted with a Macherey-Nagel polytetrafluoroethylene membrane (20 µm).

Extracts (1 mL) at each concentration were mixed with 15 mL of PDA in Petri dishes and stirred, so the extract mixed with the culture medium. After solidification, one disc of pathogen (5 mm) was transferred to the centre of each Petri dish and incubated at 27 ºC, for 15 days, under continuous



Photos: Amanda Abdallah Chaibub

Figure 1. Steps for obtaining the *W. circinata* extracts. A) Mycelial mass extract (MME): 1 - PDA for 11 days; 2 - scraped mycelia; 3 - ethyl acetate for 72 h; 4 - solvent elimination in rotary evaporator. B) Lyophilized extract (LE): 1 - PDA for 11 days; 2 - 25 dishes on PD for 15 days; 3 - vacuum filter; 4 - liquid-liquid partition with ethyl acetate (aqueous fraction); 5 - lyophilization for 24 h; 6 - ethyl acetate; 7 - solvent elimination in rotary evaporator. Crude extract (CE): 1 - PDA for 11 days; 2 - 25 dishes on PD for 15 days; 3 - vacuum filter; 4 - liquid-liquid partition with ethyl acetate (organic fraction); 5 - anhydrous sodium sulphate; 6 - ethyl acetate and vacuum filtration in Buchner apparatus; 7 - solvent elimination in rotary evaporator. C) Mycelial extract (ME): 1 - PDA for 11 days; 2 - 25 dishes on PD for 15 days; 3 - vacuum filtration; 4 - mycelial mass; 5 - lyophilization 24 h + oven 24 h; 6 - ground mycelial; 7 - ethyl acetate for 48 h; 8 - vacuum filtration; 9 - solvent elimination in rotary evaporator.

light. The treatments consisted of 1,040 µg mL^{-1}, 700 µg mL^{-1}, 520 µg mL^{-1}, 120 µg mL^{-1} and 12 µg mL^{-1} of extract, plus controls consisting of discs for each pathogen lacking extract.

The evaluations of the assays were performed when the control colony reached the edge of the dish. The horizontal and vertical diameters of pathogens were measured with a digital pachymeter to determine the colony area, and the reductions were calculated according to Carvalho et al. (2015).

To evaluate the effect of the mycelial mass extract on the conidial germination and appressorium formation, a *M. oryzae* isolate was grown and adjusted to 1×10^5 conidia mL^{-1} (Filippi & Prabhu 2001). An aliquot of 10 µL of *M. oryzae* suspensions and the mycelial mass extract (totalling 20 µL) were induced on a hydrophobic artificial surface previously sterilized under slides and kept under high humidity conditions at 27 ºC, for evaluations after 3, 6 and 24 h. The slides were observed under a light microscope and the percentage of conidial germination and appressoria formation was determined by analysing 100 conidia per replicate. The assay was conducted in a completely randomized design, with six treatments and three replicates. The treatments consisted of five mycelial mass extract concentrations (1,040; 700; 520; 120; and 12 µg mL^{-1}) and the control (*M. oryzae* without mycelial mass extract).

The leaf blast suppression by *W. circinata* mycelial mass extract was performed with two applications methods: simultaneous and previous. For the simultaneous application, rice seeds of the BRS Primavera cultivar were sown in plastic tray grooves with 3 kg of soil fertilized with NPK (5 g of 5-30-15 + Zn). Top dressing fertilization was performed at 18 days after sowing, using 3 g of ammonium sulphate per tray. The *M. oryzae* conidia production and inoculation (3×10^5 conidia mL^{-1}) were performed according to Filippi & Prabhu (2001).

Three assays were conducted in a completely randomized design, with three replications, under greenhouse conditions. Each treatment was sprayed

simultaneously with *M. oryzae* (3 x 10^5 conidia mL^{-1}) on 21-day-old rice plants (V3 stage). After growing on PDA, the *W. circinata* mycelia was scraped, weighed and diluted in autoclaved distilled water to obtain three suspensions at concentrations of 2; 5; and 10 g L^{-1}.

In the first assay, the mycelial mass and crude extracts were compared for leaf blast severity suppression. The assay was performed with three treatments each, as it follows: crude extract at 1,040 µg mL^{-1}, mycelial mass extract at 1,040 µg mL^{-1} and control. The second assay compared *W. circinata* extracts (mycelial mass and crude extracts) with mycelial suspensions for leaf blast severity, with six treatments, as it follows: mycelial suspensions at 2; 5; and 10 g L^{-1}, crude extract at 1,040 µg mL^{-1}, mycelial mass extract at 1,040 µg mL^{-1} and control. The third assay compared the optimal treatments of the second assay with the following treatments: mycelial suspensions at 5 g L^{-1}, crude extract at 1,040 µg mL^{-1}, mycelial mass extract at 1,040 µg mL^{-1} and control.

Leaf blast severity (%) was evaluated using a 10-grade rating scale (Notteghem 1981), at 8 days after the *M. oryzae* inoculation. For the first and third assays, the leaf blast severity was evaluated at 2-day intervals, to determine the area under the disease progress curve (AUDPC) (Shaner & Finney 1977). The reduction in the rice leaf blast severity was calculated according to the severity in the inoculated control (Chaibub et al. 2019).

For the previous method, the planting, inoculation and evaluation were performed according to the simultaneous application assay, but the *W. circinata* applications were performed several days after planting (DAP). Planting was carried out on October 10, 2017. The assay was conducted with 10 treatments, as it follows: 1 - soil (soil mixed with 5 g kg^{-1} of fungal disc before planting); 2 - mycelial suspension (5 g L^{-1}) at 7 DAP; 3 - mycelial suspension (5 g L^{-1}) at 14 DAP; 4 - mycelial suspension (5 g L^{-1}) at 21 DAP; 5 - mycelial suspension (5 g L^{-1}) at 7, 14 and 21 DAP; 6 - mycelial mass extract (1,040 µg mL^{-1}) at 7 DAP; 7 - mycelial mass extract (1,040 µg mL^{-1}) at 14 DAP; 8 - mycelial mass extract (1,040 µg mL^{-1}) at 21 DAP; 9 - mycelial mass extract (1,040 µg mL^{-1}) at 7, 14 and 21 DAP; 10 - control. The mycelial suspension and mycelial mass extract were added to the soil at 50 mL/3 kg of soil.

For all the assays, analyses of variance and the Tukey test ($p < 0.05$) were performed using the R software, version 3.0.1 (R Core Team 2019), and all the data from the greenhouse assays were transformed ($\sqrt{x} + 0.5$).

RESULTS AND DISCUSSION

The mycelial mass extract at 1,040 µg mL^{-1} inhibited the colony area of *C. miyabeanus*, *M. albescens* and *S. oryzae* by 33, 12.5 and 51 %, respectively (Figures 2B, 2D and 2F). The crude (120 and 12 µg mL^{-1}) and lyophilized (520 µg mL^{-1}) extracts inhibited *C. miyabeanus* by 29.40 and 29.49 %, respectively, and *M. albescens* by 26.5 % (Figures 3A and 3F). Carvalho et al. (2015) showed that the crude extract (700 µg mL^{-1}) is the only extract that inhibits the mycelial growth of *M. oryzae* by 75 %.

The mycelial mass extract was obtained in a few steps and 14 days (Figure 1A), while the lyophilized (Figure 1B), crude (Figure 1B) and mycelial (Figure 1C) extracts demanded a high number of stages and 30, 27 and 26 days, respectively (Figure 1). Therefore, among the tested extracts, the mycelial mass extract showed the best cost-benefit ratio, because it is faster to obtain, uses less solvent and needs minor amounts of PDA. All the extracts studied by mass spectrometry have sugar adducts and are the major interference by ion suppression on presence of saccharides adducts derivatives, but the new extract has minor interferences, because it has less amounts of PDA.

Côrtes et al. (2014) also demonstrated that the *S. oryzae* crude extract inhibits the *M. oryzae* mycelial growth by 79 %, and Dethoup et al. (2018) detected the inhibition of four rice pathogens (*Alternaria padwickii*, *Bipolaris oryzae*, *Curvularia lunata* and *Fusarium moniliforme*) using *Talaromyces tratensis* crude extract.

Therefore, a more sensitive and accurate test with the mycelial mass extract was performed to verify its potential for inhibiting the conidial germination and appressorium formation for the main rice pathogen (*M. oryzae*).

The tested concentrations of mycelial mass extract affected the germination and appressorium formation of *M. oryzae* at different times. The mycelial mass extract (1,040 µg mL^{-1}) inhibited the *M. oryzae* conidial germination by 59.22 and 74.50 % and the appressorium formation by 44.74 and 85.86 %, at 4 and 6 h after the contact,

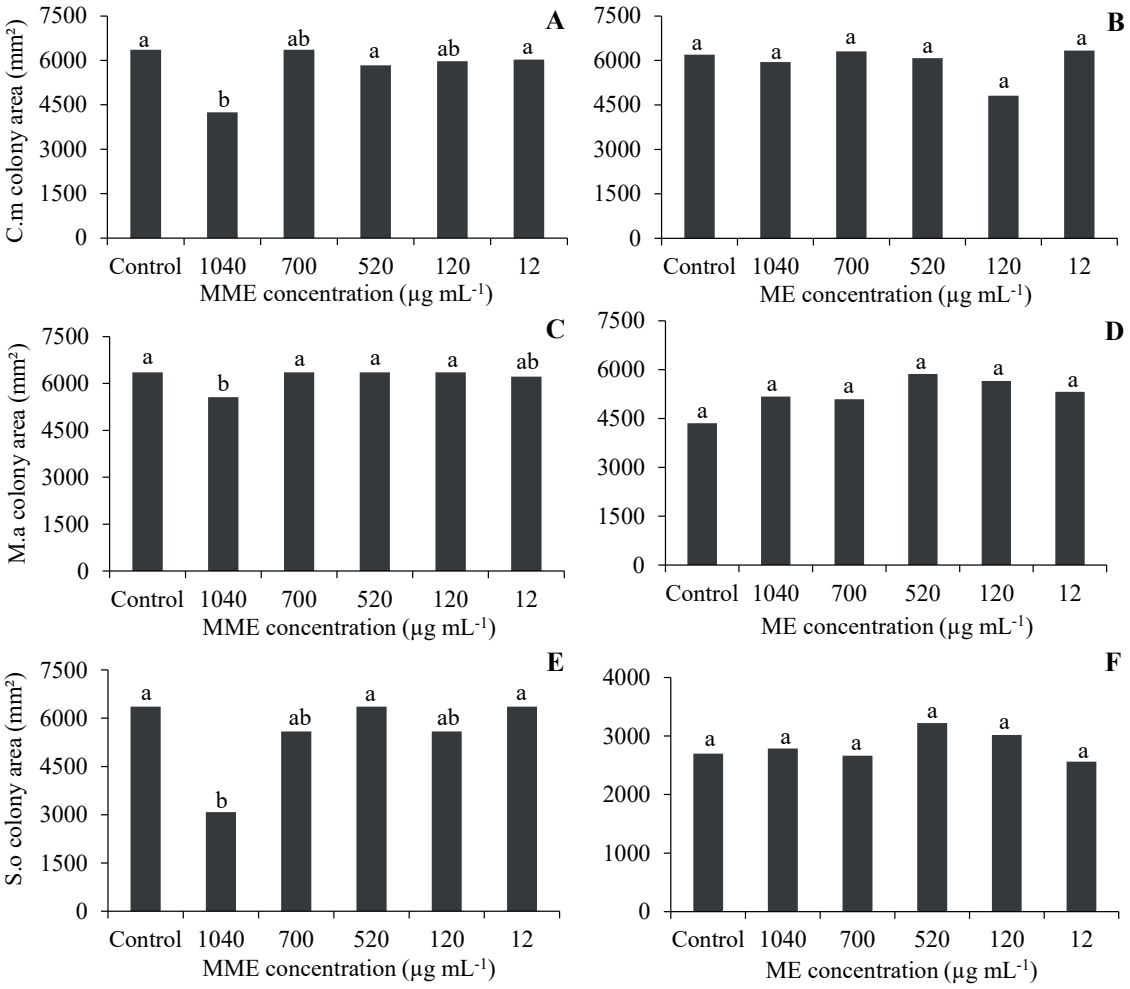


Figure 2. Inhibition of mycelial growth of rice pathogens by mycelial (ME) and mycelial mass (MME) extracts of *W. circinata*. C.m: *Cochliobolus miyabeanus*; M.a: *Monographella albescens*; S.o: *Sarocladium oryzae*. Means followed by the same letter were not significantly different from each other, according to the Tukey test ($p < 0.05$).

respectively. Carvalho et al. (2015) reported that the crude, lyophilized and mycelial extracts inhibit the conidial germination at 24 h by up to 31 %, and that the crude extract inhibits the *M. oryzae* appressorium formation by 100 % at 3, 6 and 24 h (Figure 4). Although Carvalho et al. (2015) detected up to 100 % of inhibition for appressorium formation by the crude extract, the present study detected a higher conidial germination inhibition (74.50 %) in the first hours after the contact with the mycelial mass extract (4 and 6 h), and the appressorium formation was reduced in the first hours after the contact with the mycelial mass extract. Therefore, the mycelial mass extract concentration of 1,040 µg mL^{-1} is the most recommended one, because its best effect occurred in the initial hours of contact with *M. oryzae*, interfering in the beginning of the pathogen cycle. Cortês et al. (2014) also demonstrated that *S. oryzae* crude extract containing cerulenin inhibits the germination and appressorium formation of *M. oryzae* by 98 % and 99 %, respectively, at 24 h.

For the simultaneous application, in the first assay, the mycelial mass and crude extracts showed 0.13 and 0.59 % of leaf blast severity and 2.13 and 3.18 of AUDPC, when compared to the control, which presented 18.86 % of leaf blast severity and 27.83 of AUDPC (Figure 5).

In the second assay, the treatments containing the mycelial suspensions, crude extract and mycelial mass extract reduced the leaf blast severity, with values of 4.50, 5.22 and 5.24 %, respectively, when compared to the control (16.38 %) (Figure 6).

The third assay showed that the AUDPC and the leaf blast severity were reduced by the mycelial

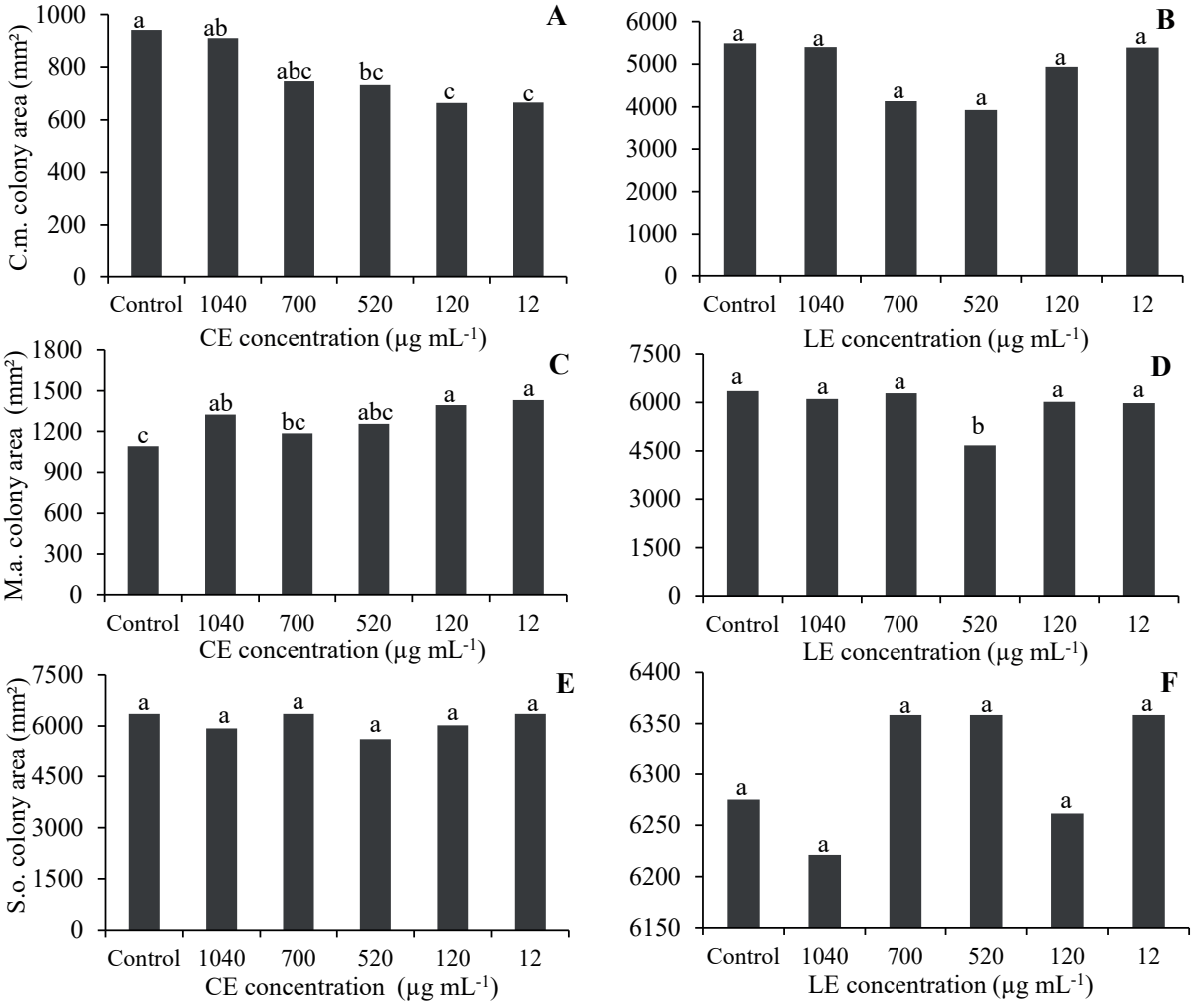


Figure 3. Inhibition of mycelial growth of rice pathogens by crude (CE) and lyophilized (LE) extracts of *W. circinata*. C.m: *Cochliobolus miyabeanus*, M.a: *Monographella albescens*; S.o: *Sarocladium oryzae*. Means followed by the same letter were not significantly different from each other, according to the Tukey test ($p < 0.05$).

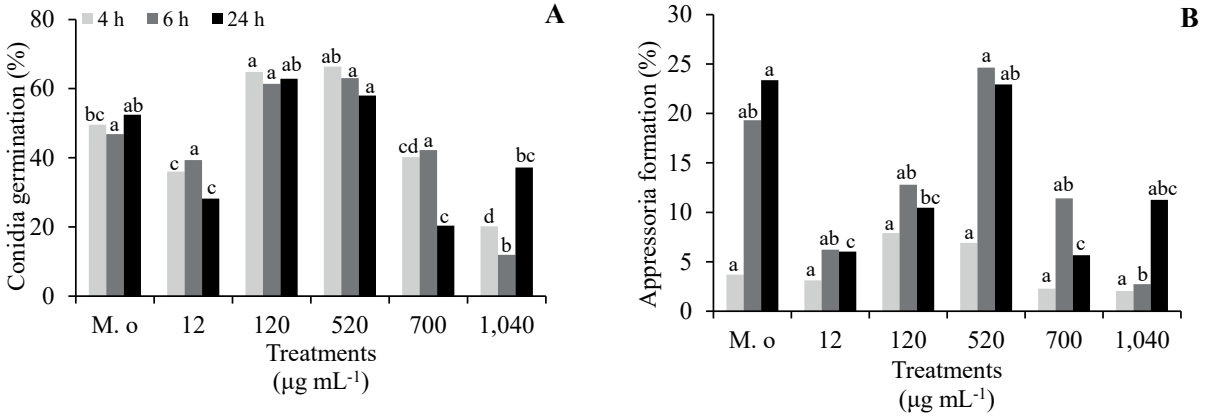


Figure 4. Effect of mycelial mass extract (MME) at 12, 120, 520, 700 and 1,040 µg mL^{-1} against *Magnaporthe oryzae* (M.o) without mycelial mass extract (control). A) *M. oryzae* conidia germination; B) appressoria formation at 4, 6 and 24 h after the conidial deposition on the surface. Means followed by the same letter were not significantly different from each other, according to the Tukey test ($p < 0.05$).

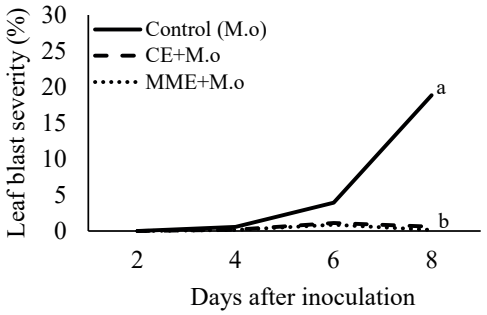


Figure 5. Leaf blast severity and area under the disease progress curve in rice plants treated with crude (CE) and mycelial mass (MME) extracts at 1,040 µg mL^{-1} and control inoculated only with *Magnaporthe oryzae* (M.o). Means followed by the same letter were not significantly different, according to the Tukey test ($p < 0.05$).

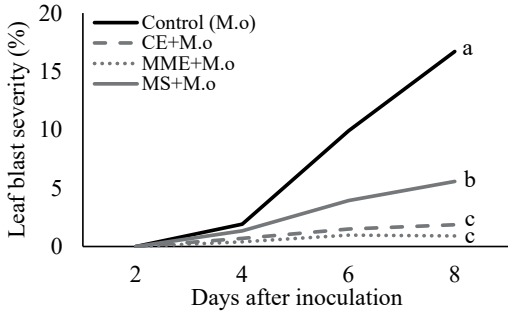


Figure 7. Leaf blast severity and area under the disease progress curve, in rice plants treated with mycelial suspension (MS) at 5 g L^{-1}, crude (CE) and mycelial mass (MME) extracts at 1,040 µg ml^{-1}, when compared with the control inoculated only with *Magnaporthe oryzae* (M.o). Means followed by the same letter were not significantly different, according to the Tukey test ($p < 0.05$).

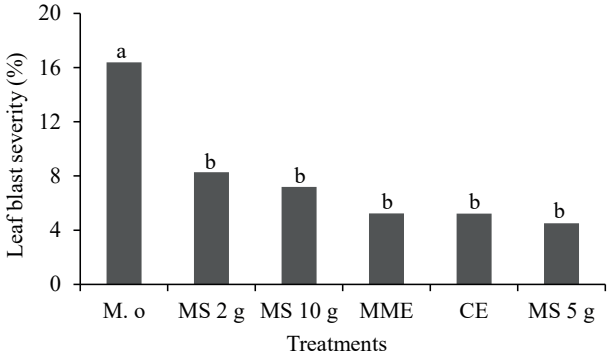


Figure 6. Leaf blast severity of rice plants treated with crude (CE) and mycelial mass (MME) extracts at 1,040 µg mL^{-1} and mycelial suspensions (MS) at 2, 5 and 10 g L^{-1}, when compared with the control inoculated only with *Magnaporthe oryzae* (M.o). Means followed by the same letter were not significantly different, according to the Tukey test ($p < 0.05$).

suspension (5 g L^{-1}; 5.58 and 16.16 %), crude extract (1.87 and 6.31 %) and mycelial mass extract (0.91 and 3.72 %), when compared to the control (16.71 and 40.4 %) (Figure 7). The treatments with 5 g L^{-1} of mycelial suspensions and mycelial mass extract suppressed the leaf blast by 72 and 68 %, respectively. When the effectiveness of 5 g L^{-1} of mycelial suspensions, crude extract and mycelial mass extract were compared, the AUDPC was reduced by 60, 85 and 91 %, respectively. In a study by Carvalho et al. (2015), the crude extract reduced the AUDPC by 25 %, at eight days after the inoculation with *M. oryzae*, using mycelial suspensions. Dethoup et al. (2018) showed brown spot and dirty panicle reductions of 56.74 and 60 % in rice, after treatment with crude extract and conidial suspension of *T. tratensis*, respectively.

The effect of the different extracts (crude and mycelial mass extracts) is probably due to the composition of the metabolites, that may act synergistically or not, cultivation of the bioagent and how to obtain it (El-Hossary et al. 2017, Dethoup et al. 2018).

For the previous application, all the treatments reduced the leaf blast severity, when compared to the control, except for the mycelial mass extract at 14 DAP. The treatments mycelial suspension at 14 DAP, mycelial mass extract and mycelial suspension at 7, 14 and 21 DAP, and mycelial mass extract at 21 DAP showed a lower leaf blast severity, with values of 2.95, 2.23, 2.22 and 1.81 %, respectively, when compared to the control (30.6 %) (Figure 8).

Promising results have also been obtained with previous applications. The suppression of leaf blast by soil mixed with fungal discs reached 84 %, if compared to the control. For the treatments applied on the soil by drenching with mycelial suspension, the suppression percentages were 82 % (7 DAP), 90 % (14 DAP), 24 % (21 DAP) and 93 % (7, 14 and 21 DAP). The suppression percentages, when mycelial mass extract was applied on soil by drenching, were 62 % (7 DAP), 94 % (21 DAP) and 93 % (7, 14 and 21 DAP). These findings indicate that *W. circinata* and its extracts may have more than one mechanism against rice blast, corroborating the results by Xu et al. (2016).

We hypothesized that the previous application of both the fungus and extracts on the soil was due

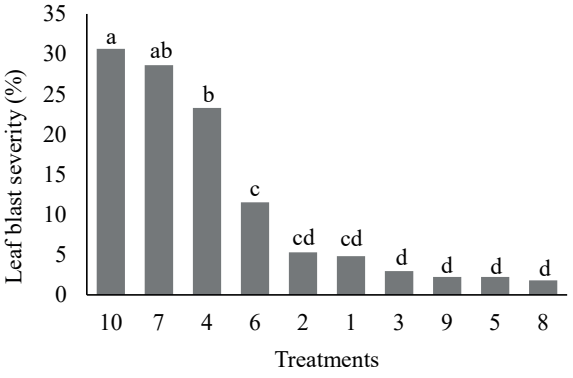


Figure 8. Leaf blast severity of rice plants treated with: 1 - soil (soil mixed with 5 g kg^{-1} of fungal disc before planting); 2 - mycelial suspension (5 g L^{-1}) at 7 days after planting (DAP); 3 - mycelial suspension (5 g L^{-1}) at 14 DAP; 4 - mycelial suspension (5 g L^{-1}) at 21 DAP; 5 - mycelial suspension (5 g L^{-1}) at 7, 14, and 21 DAP; 6 - mycelial mass extract (1,040 µg mL^{-1}) at 7 DAP; 7 - mycelial mass extract (1,040 µg mL^{-1}) at 14 DAP; 8 - mycelial mass extract (1,040 µg mL^{-1}) at 21 DAP; 9 - mycelial mass extract (1,040 µg mL^{-1}) at 7, 14 and 21 DAP; 10 - control (inoculated only with *Magnaporthe oryzae*). Means followed by the same letter were not significantly different, according to the Tukey test ($p < 0.05$).

to a resistance inducer, because the pathogen was presented on the leaf surface and *W. circinata* is a root inhabitant. The simultaneous leaf application, however, showed an antibiosis effect, due to the action of toxic compounds directly on the pathogen. Similarly to the present study, Chaibub et al. (2019) demonstrated that the leaf blast severity reaches 83.78 %, when *Cladosporium cladosporioides* is administered at 24 h or 48 h before *M. oryzae*.

The *W. circinata* mycorrhizal fungus is a promising option for formulations of bioproducts, as it is a non-mandatory symbiotic, what facilitates its large-scale production. Besides, in a previous study, *W. circinata* increased the rate of symbiotic germination *in vitro* by 81 % (Carvalho et al. 2015). This effect is due to the increase in water and nutrient uptake (Rasmussen & Rasmussen 2009), which act as antagonists to plant pathogens (Mosquera-Espinosa et al. 2013, Carvalho et al. 2015).

Therefore, this study of the *W. circinata* effects and its extracts and metabolites against important rice pathogens will allow the inclusion of a single biocontrol agent adapted to the *Cerrado* (Brazilian Savanna) conditions in the rice disease control management.

CONCLUSION

The new mycelial mass extract showed to be the most efficient one for inhibiting the mycelial growth of rice pathogens and suppressing rice blast, when compared to the other extracts; therefore, it has a potential for use in rice fields, reducing the use of fungicides and promoting a more sustainable agriculture.

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